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Laparoscopy in Hodgkin's Disease

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Abstract The value of systematic laparoscopy for the definition of the illness stage in every case of initial Hodgkin's disease is shown

Key Words
Hodgkin's disease
Laparoscopy
Liver biopsy

We usually clinically assume the localization of Hodgkin's disease with a cranio-caudal direction apparently involving the superficial and deep lymphnodes It is generally admitted that early and wide removal of tissues affected by the granulomatous process can offer long periods of remission and sometimes even clinical recovery It is therefore essential to perform a wide range of tests in order to identify the disease at its earliest stage

Radiological techniques are important to identify eventual involvements of the deep lymphatic stations which cannot be revealed by clinical examination The isotope techniques are useful to reveal liver and or spleen localization [2, 16, 17, 19] Unfortunately the clinical methods alone, even though correctly applied, are not always adequate, and failure of remission of the disease after surgical removal of the primitive localization may occur

Forms of Hodgkin's disease with early deep mediastinal [1, 4, 8, 13] or subdiaphragmatic localization are well known [5] The onset of the disease by involvement of the intestinal lymph glands is also not exceptional [3, 9, 15, 18]

In the above-mentioned cases it is obvious that an enlargement of the cervico-axillary and mediastinal lymph nodes may be wrongly interpret-

Table I

Case No	Age, years	Sex	Stage	Liver scintigraphy	Lymphangio-graphy	Alkaline phosphatase (9–35 I U)	BSP (max 5%/ 45 min)	PPD test	Laparoscc
1	33	M	II	neg	neg	21	3	+ − −	neg
2	37	M	II	n p	neg	15	2	neg	neg
3	30	F	I	n p	n p	5	3	+ − −	neg
4	49	M	I	n p	n p	13	4	neg	neg
5	25	M	II	n p	n p	27	5	neg	neg
6	42	M	II	neg	n p	30	2	neg	neg
7	41	F	II	neg	neg	22	3	neg	neg
8	29	F	II	neg	neg	30	3	+ − −	neg
9	38	M	II	n p	n p	32	3 5	neg	neg
10	22	F	II	n p	n p	28	5	neg	neg
11	51	M	II	n p	n p	26	3	+ − −	neg
12	53	M	II	neg	neg	23	3	neg	neg
13	66	M	II	neg	neg	22	2	neg	neg
14	29	F	I	neg	neg	20	2	+ − −	pos
15	32	F	I	neg	neg	18	3	+ − −	neg
16	47	M	II	neg	neg	10	2	neg	neg
17	20	M	II	neg	neg	14	2 5	neg	neg
18	26	F	II	neg	neg	21	4	neg	neg
19	35	M	II	n p	n p	28	3	neg	neg
20	45	F	II	n p	n p	25	2	neg	neg
21	50	M	II	n p	n p	30	5	neg	neg
22	46	M	I	n p	n p	21	3	+ − −	neg
23	43	F	II	neg	neg	25	1 5	neg	pos
24	31	F	II	neg	neg	27	2	neg	neg
25	34	M	II	neg	neg	19	5	neg	neg
26	28	M	II	neg	neg	30	5	neg	neg
27	30	M	II	neg	neg	27	3	+ − −	neg
28	34	F	II	neg	neg	22	2	neg	neg
29	45	F	II	n p	n p	30	2	neg	neg
30	20	F	II	neg	neg	30	3	neg	neg
31	48	M	I	n p	n p	19	5	+ + −	neg
32	22	M	II	n p	neg	22	5	neg	neg
33	29	M	II	neg	neg	30	4	neg	neg
34	33	M	II	neg	neg	27	2	neg	neg
35	19	M	II	neg	neg	25	5	neg	neg
36	30	M	II	neg	neg	24	5	neg	neg
37	18	F	I	n p	n p	20	4	+ + −	neg
38	20	M	II	neg	neg	20	8	neg	neg

Pos = positive, neg = negative, n p = not performed

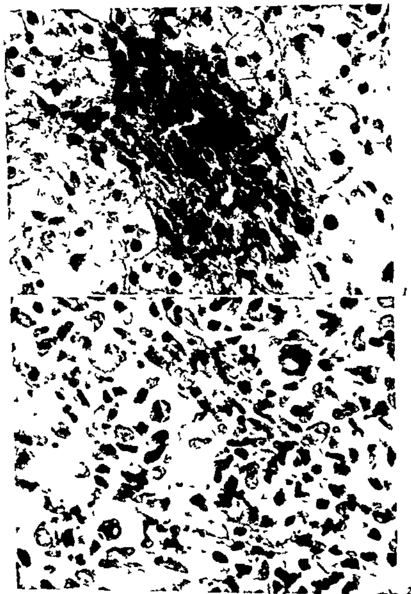


Fig 1
Fig 2

ed as primary localization In this case it would be incorrect to extirpate the cervico axillary lymph nodes

It is the general opinion that hepatic involvement of the malignant granuloma occurs in a late stage and that it indicates an invasion of the granulomatous process throughout the body Some references report cases of Hodgkin's disease beginning with jaundice, meaning a hepatic involvement of the malignant granuloma [3, 4, 10, 12, 20, 21] It is therefore important to complete the routine tests with liver and spleen scanning laparoscopy and liver biopsy

Throughout many years we have performed laparoscopy or liver biopsy in patients admitted to the Medical Clinic of the University of Pavia with the diagnosis of first or second stage Hodgkin's disease Our investigation made us aware, by direct vision of the actual hepatic involvement In 38 cases (table I) we had no clinical evidence or suspicion of involvement Only in one case, clinically classified as a second stage, a single little granulomatous lesion at the medial aspect of the right lobe near the suspensory ligament could be demonstrated (fig 1) The clinical course was so tumultuous and rapid however, that we suggested a transitional evolution of the disease between the second and third stage In another patient instead, clearly classified as a second stage, the laparoscopy showed on the interior surface of the right lobe, where the liver colour had a grey shade, granulomas without Reed Sternberg cells (fig 2) Clinical observation at a later time showed an early liver enlargement

It is concluded that in only 2 cases we have demonstrated an early liver involvement, this means about 5% of all cases examined Our observation is in agreement with that of LEVITAN *et al* [13] and we think that the routine application of laparoscopy is justified in the group of clinical tests commonly used for the diagnosis of Hodgkin's disease and the assessment of its stages of evolution

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La cinétique des myéloblastes leucémiques humains après cytosine-arabinoside

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Abstract Perturbations of the generation cycle of human leukemic myeloblasts *in vivo* by cytosine arabinoside were studied by *in vitro* labeling of human bone marrow with tritiated thymidine Ten patients with myeloblastic acute leukemia at onset of the disease or in relapse received a 4 h infusion of the drug Mitotic and labeling indexes were calculated before and after chemotherapy A kinetic pattern indicating a specific action of the drug on the S phase lasting about 20 h was found A secondary increase of the labeling index beginning 24 h after treatment was observed The results suggest a synchronization of the blastic cells in cycle 48 h after cytosine arabinoside injection

Key Words
Autoradiography
Cell kinetics
Cytosine arabinoside
Generation cycle
Leukemia therapy
Myeloblastic leukemia

Nous avons étudie les modifications du cycle cellulaire des cellules myeloblastiques après perfusion de 4 h d une dose unique de cytosine-arabinoside Cette etude a été pratiquée chez 10 sujets atteints de leucémie aiguc, soit en debut de maladie soit en rechute Nous basant sur des données experimentales obtenues tant chez la souris [20] que chez l'etre humain [14–16] nous avons essaye dans un premier temps de determiner la durée d action de la cytosine sur la phase S, le moment et l intensite de la reprise afin de voir s'il était possible dans un 2e temps d en tirer des arguments en vue d un schema therapeutique tenant compte du moment d'une éventuelle synchronisation

Matériel et methodes

Les 10 sujets étudies avaient une leucemie aigue myéloblastique avec envahissement medullaire supérieur à 50% (tab I)

[1] Avec la collaboration technique de F Maroulle

Tableau I Principales données cliniques Leucemies aiguës myéloblastiques

Cas No	Sexe	Age, ans	Moment de la maladie	Blastose medullaire %	Cytologie sanguine Hb g%	leuco-cytes/ mm³	blastes %
1	M	36	debut	98	7,75	46 000	86
2	M	34	debut	91	7 0	91 200	94
3	M	30	debut	70	11,25	4 000	0
4	F	5	1re rechute 7 mois après remission complète	60	11,75	4 000	16
5	M	6.5	1re rechute 7 mois après rémission complète	52	11,0	2 400	2
6	F	32	début	80	11,0	1 800	0
7	F	18	debut	97	10,25	40 000	82
8	F	14	1re rechute 5 mois après remission complète	50	10 50	32 000	22
9	M	42	debut	52	7,50	32 800	21
10	F	29	début	98	8 0	2 200	90

Un prem [illegible]

[illegible] été faits toutes les 24 h jusqu à la 144e heure pour le 3e patient. Ce dernier a, de plus, reçu une seconde perfusion de cytosine après le prelevement de la 72e heure

Un deuxième protocole a été appliqué à 7 patients. Pour ces derniers la dose de CA a été de 5 mg/kg et les prélèvements ont été faits avant perfusion de CA et 48 h après

Sur chaque prélèvement on a calculé la valeur de l index de marquage et de l index mitotique des cellules blastiques

L index de marquage a été determiné d apres un decompte fait sur 2000 cellules blastiques après incubation *in vitro* de la moelle avec de la thymidine tritiee (^{3}H Tdr) Le prélèvement médullaire recueilli sur anticoagulant (EDTA disodique à 5%) est mis à incuber $^1/_2$ h à 37 °C en presence de thymidine tritiee (^{3}H Tdr, activité spécifique 5–10 Ci mn, 5–10 μCi ml) Après centrifugation à faible vitesse étalement des frottis et fixation au méthanol pur ($^1/_2$ h), l exposition dure 8 jours (Emulsion Dipping K 2 Ilford) Les lames sont alors revelées (Kodak D 19B), fixées (Kodak A L4) puis colorées au Giemsa

Tableau II Modifications de l index de marquage (LI) et de l index mitotique (MI) de la population blastique en fonction du temps après injection de CA

Cas No	Index	0 h	6 h	24 h	48 h	72 h	96 h	120 h	144 h
1	LI, %	6,7	1,10	3,8					
	MI, ‰	2 7	0 6	1					
2	LI, %	4 5	1	1,3					
	MI, ‰	0,6	0	0,3					
3	LI, %	5,8	0,10	4,9	5	4 5	4,9	4,3	4,6
	MI, ‰	1,7	0,3	0	0	0,6	0	0,6	0 6
4	LI, %	11	–	–	16,3				
	MI, ‰	2,3	–	–	5,7				
5	LI, %	8,5			10,8				
	MI, ‰	1			2,3				
6	LI, %	8,3			13,6				
	MI, ‰	1			2				
7	LI, %	12,2			13,8				
	MI, ‰	2,7			4,4				
8	LI, %	9,5			14				
	MI, ‰	–			–				
9	LI, %	12			10				
	MI, ‰	–			–				
10	LI, %	5,2			3				
	MI, ‰		–		–				

L'index mitotique a été déterminé sur des frottis de moelle avant toute manipulation, d'après le décompte de 3000 blastes Les frottis étaient colorés au May-Grunwald Giemsa

Résultats

Chez les 3 sujets du premier protocole (prélèvements avant traitement à la 6e et la 24e heure) l'index de marquage est quasi nul à la 6e heure puis remonte a la 24e heure a des valeurs proches des valeurs de départ, l'index mitotique suit une évolution superposable (tab II), l'index de marquage ne dépasse pas le taux initial, chez le sujet 3, lors des prélèvements plus tardifs (tab II) Par contre, dans le deuxième protocole (prélevements avant traitement et la 48e heure après), on note une aug-

mentatıon nette de l ındex de marquage a la 48e heure chez 5 des 7 patıents etudıes Par rapport au marquage ınıtıal prıs comme unıte l aug mentatıon est de 1 14 1 28 1 45 1 46 et 1 65 L ındex mıtotıque suıt une evolutıon parallele Pour les 2 sujets restant, l ındex de marquage dımınue à la 48e heure L absence de prelevements ıntermedıaıres entre l ınjectıon et 48 h et de prelèvements tardıfs apres 48 h rend ces 2 exa mens dıffıcılement ınterprétables car une augmentatıon transıtoıre de l ın dex de marquage a pu se produıre plus précocement ou plus tardıvement Ces deux sujets aınsı que le cas 3 temoıgnent de l heterogéneıte des reac tıons medullaıres à la CA deja soulıgnée par certaıns auteurs [16] Cepen dant la majorıté des malades étudıes evoluent de façon sımılaıre

Les tableaux I et II resument les donnees clınıques et cınétıques des 10 cas etud es

Dıscussıon

Actıon de la CA sur la prolıferatıon La CA ne dıffere chımıquement de la cytıdıne que par le remplacement du rıbose par l arabınose soıt au total une sımple ınterversıon des groupes hydrogene et hydroxyle au nıveau de l atome C_2 Cette parente bıochımıque luı permet de se substı tuer à la molécule de cytıd ne en empechant la formatıon de la deoxycytı dıne trıphosphate [2] quı est un substrat de la DNA polymerase Des étu des *ın vıtro* [10] et des experımentatıons anımales [10] et humaınes ont confırmé cette actıon bıologıque [7 21] et ont permıs de verıfıer l actıon spécıfıque de cette drogue au nıveau de la phase S (arret de toute synthese d ADN) dans le cycle cellulaıre [9–11]

S ıl s ag t bıen d une drogue specıfıque de la phase S on peut en atten dre les effets suıvants (6)

(1) Une chute rapıde et brutale de l ındex de marquage puısque les cel lules sont bloquées dès leur entree en S La chute de l'ındex de marquage que nous obtenons à la 6e heure est en accord avec les expérımentatıons anımales [10] et confırme bıen la specıfıte d actıon de la cytosıne sur cette phase du cycl[illegible]

les temps (t 0 [illegible]

nus tant dan [illegible] que dans les leucemıes humaınes [14–16] que l ınhıbıtıon de la synthèse d ADN est quası totale dès la 2e heure

(2) Une dım nutıon de l ındex mıtotıque apres un temps correspondant à la durce du trans t cellulaıre au travers de la phase G_2 Dans notre

expérience, l'index mitotique est nul à la 6e heure. Dans le travail de LAMPKIN *et al.* [16] il est également très bas dans les prélèvements des 3e ou 4e heures.

Si la CA est étroitement spécifique de la phase S, l'effondrement de l'index de marquage doit précéder de la durée de G_2 l'effondrement de l'index mitotique. Les faits expérimentaux [16, 17] indiquent qu'après injection de CA l'index de marquage s'effondre dès la 1re heure, d'autre part, la chute de l'index mitotique n'est observée qu'après la 150e minute. La CA est donc bien, au sens cinétique du terme, une drogue spécifique de la phase S des cellules en cycle.

Reprise de la prolifération. Le moment après lequel reprend la prolifération est fonction de 2 paramètres de temps essentiels: (a) la durée pendant laquelle la concentration sanguine de la drogue est au niveau efficace, (b) la durée de l'inhibition enzymatique dans la cellule-cible.

Le temps de demi-disparition de la CA est très court [23], la durée de l'inhibition est plus difficile à déterminer d'autant que la cytosine n'agit pas directement mais après phosphorylation et est inactivée par déamination [3, 5, 12, 23]. Chez l'animal, la récupération de l'activité de synthèse d'ADN semble toujours se faire dès la 6e heure [17]. Dans les 3 observations où nous avons pu étudier l'index de marquage à la 24e heure, il était toujours très nettement supérieur à celui de la 6e heure, ce qui s'accorde aux faits de la littérature [16].

Synchronisation et recrutement. Toute action d'une drogue spécifique de la phase S peut faire espérer une reprise de prolifération avec un certain degré de synchronisation. Le moment de la synchronisation en S serait alors choisi pour administrer la 2e dose de la drogue spécifique de cette phase du cycle, augmentant de ce fait son efficacité.

Dans notre deuxième protocole, l'augmentation de l'index de marquage est évidente à la 48e heure. Par rapport au marquage initial, les valeurs sont respectivement de 1,14, 1,28, 1,45, 1,46 et 1,65. Si l'on admet un temps de S de 20 h [13] et un temps de cycle de 40 h [13] environ pour la fraction de la population blastique en cycle, après blocage des cellules au début de la phase S par la CA, une synchronisation des cellules en cycle ne pourrait augmenter l'index de marquage que de 1 à 2, la multiplication par 2 impliquant que toutes les cellules en cycle sont en phase S. De ce fait on peut considérer les valeurs observées de 1,45 et au-delà comme l'indice d'une synchronisation certaine. Des valeurs semblables sont retrouvées dans la littérature [15, 16]. Le moment de la 'synchronisation' est différent d'un sujet à l'autre dans nos cas comme dans

ceux de la littérature [16] retrouvé à des temps variables de la 24e à la 48e heure Toutefois le nombre limité de points expérimentaux ne permet pas d exclure une meilleure synchronisation antérieure ou postérieure aux dates auxquelles les prélèvements de moelle ont été faits

L analyse des observations de LAMPKIN [16] montre dans certains cas une montée tardive et importante de l index de marquage à la 72e ou à la 84e heure avec parfois un chiffre atteignant 5 fois la valeur initiale Si on accepte un temps moyen de cycle de 40 h pour les cellules leucémiques humaines en cycle on peut raisonnablement penser qu une montée tardive et aussi intense ne peut s expliquer que par un recrutement de cellules précédemment hors cycle

Le phénomène de recrutement peut être obtenu après une dose initiale d une drogue ayant éventuellement entraîné une synchronisation partielle préalable Nos résultats personnels ne nous permettent aucun progrès concernant cette idée Un protocole expérimental cherchant le moment d un recrutement en cycle éventuel des cellules leucémiques est en cours

L index mitotique suit une évolution très peu différente (compte tenu du nombre limité de points expérimentaux) de celle de l index de marquage Il s effondre à la 6e heure et ébauche une remontée à la 24e heure Si la dose de drogue spécifique de S est suffisante pour tuer les cellules présentes dans cette phase au moment de son administration l augmentation de l index de marquage contemporaine de la levée de l inhibition doit être suivie d une augmentation de l index mitotique retardée de la durée de S + G_2 Une telle observation pourrait être déduite de certaines courbes de LAMPKIN [16] Nos documents personnels ne le confirmant pas bien que les doses utilisées aient été les mêmes ceci suggère qu usuellement de telles doses sont davantage cytostatiques que cytolytiques

Intérêt de la recherche d une synchronisation et d un recrutement Trois notions sont à la base des recherches thérapeutiques tenant compte de faits cinétiques (1) Couverture de cycle c est à-dire administration d une drogue spécifique de cycle dans une séquence telle que toutes les cellules-cible traversent la phase du cycle pendant ce temps (2) *Synchronisation* c est à-dire obtention d un taux élevé de cellules dans une phase du cycle à un temps déterminé où une drogue spécifique de cette phase *pourrait être administrée* (3) *Recrutement* c est à-dire mise en activité proliférative de cellules précédemment hors cycle donc non sensibles jusqu alors aux drogues spécifiques des cellules en cycle

Chez la souris de nombreuses expériences ont été tentées les essais les plus prometteurs paraissant être ceux de SKIPPER [19-21] basés sur la

notion de couverture de cycle. Chez la souris L 1210, l'administration de CA toutes les 3 h pendant 24 h aux jours 2, 6, 10 et 14 après transplantation, permet de guérir l'animal. Les essais thérapeutiques menés chez l'homme en tenant compte de la notion de couverture de cycle [8, 18, 22] n'ont apporté aucun élément encourageant, mais ils ne tenaient pas compte du fait que, contrairement à la leucémie murine, les leucémies humaines comportent une large population de cellules blastiques hors cycle.

Des explorations plus intéressantes ont été conduites chez l'homme lors d'essais de synchronisation [1, 16]. Là aussi, malgré une synchronisation objectivée par l'index de marquage et l'index mitotique [1, 16], le bénéfice clinique n'a pas été très net. De tels échecs paraissent explicables dans la mesure où la séquence thérapeutique se fonde sur la synchronisation partielle d'une population leucémique dont en moyenne moins de 20% des cellules sont en cycle. Il serait intéressant de trouver un protocole permettant de recruter en cycle le maximum de cellules leucémiques et de n'appliquer qu'à ce moment-là l'essai de synchronisation.

Conclusion

La CA est bien une drogue spécifique de la phase S et elle permet d'obtenir une synchronisation partielle de la population leucémique. L'absence de résultats thérapeutiques obtenus par les essais de synchronisation à l'aide de ce produit chez l'homme semble être due au fait qu'elle a été appliquée à une population très peu proliférante. Rappelons à ce propos que les expérimentations animales [20] ne permettent de guérir la leucémie L 1210 que si l'animal a un taux relativement peu élevé de cellules leucémiques (10^6). Si les mêmes essais sont entrepris à 10^9 cellules leucémiques, on ne peut espérer guérir l'animal qu'après avoir ramené sa population leucémique globale à 10^6 par une drogue non spécifique de cycle. Il en est de même pour les blastes en culture [4]. Si la culture est en phase de croissance logarithmique, aucune cellule ne survit après 96 h d'exposition continue à la drogue, si elle est en phase stationnaire, la croissance peut reprendre même après 10 jours d'exposition continue. Toute drogue spécifique de cycle appliquée à une population peu proliférante demande donc à être administrée en un temps correspondant à 5 cycles moyens de la population en cycle (soit perfusion > que 10 jours pour les leucémies humaines). Tout essai de synchronisation ne peut donc

être tenté avec quelque chance de succes qu'après recrutement préalable d une partie de la population hors cycle

Resumé

Nous avons étudie les modifications du cycle cellulaire des myéloblastes leucémiques humains apres perfusion unique de CA chez 10 sujets ayant une leucemie aiguë myeloblastique, soit en debut de maladie, soit en rechute Nous avons calcule les modifications, en fonction du temps, de l index de marquage et de l index mitotique après administration de la drogue Nous avons pu verifier ainsi la spécificité d action de la CA au niveau de la phase S du cycle cellulaire, étudier sa duree d action, objectiver le moment et l intensité de la reprise cellulaire Cette dernière se fait vers la 24e heure avec une synchronisation plus ou moins importante à la 48e heure Ces résultats peuvent aider à trouver de meilleures séquences de temps pour l application d un schéma thérapeutique utilisant une drogue spécifique de la phase S des cellules en cycle

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'Latent' Pernicious Anaemia in Delhi (India)

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Abstract Parietal cell antibody vitamin B_{12} and pepsinogen in serum were measured for the investigation of prevalence of pernicious anaemia amongst Indians in the Delhi area in India Low levels of serum pepsinogen were observed in age groups 11–20 and 41–50 years and low levels of serum pepsinogen appear to occur with greater frequency in vitamin B_{12} deficient subjects The significance of these findings in relation to the prevalence of pernicious anaemia is discussed

Key Words
Gastric mucosa antibodies
Latent pernicious anaemia
Pepsinogen
Pernicious anaemia in India
Vitamin B_{12}

The prevalence of pernicious anaemia amongst the people originating from the countries of northern Europe is well established [5, 12, 21] It is generally considered rare in Indians and the population of other Asian countries Nevertheless, a number of well-documented cases of pernicious anaemia in Indians and other Asians have been described in recent years [3, 6 9 15], which suggest that this disease is likely to be more prevalent than has been recognized so far

The problem of recognition of pernicious anaemia in India and other Asian countries is made considerably difficult by more commonly occurring megaloblastic anaemias in association with malnutrition and tropical sprue The association of gastric atrophy, parietal cell and intrinsic factor antibodies with pernicious anaemia has been well studied in western countries and these parameters have been used to examine the prevalence of latent pernicious anaemia in these countries Though a biopsy of gastric mucosa is required for the confirmatory diagnosis of gastric atrophy an indirect assessment of the peptic cell mass can be made by

determining serum levels of pepsinogen – a precursor enzyme of pepsin which is secreted mainly by the peptic cells of the stomach.

It has been recognized for some time that the peptic cells of the stomach secrete considerable amount of pepsinogen in the blood stream, which can be measured directly by relatively simple biochemical techniques. Previous workers have examined the relationship between serum or plasma levels of pepsinogen and the status of parietal and peptic cell mass of the stomach, and have shown low levels of pepsinogen in presence of gastric atrophy [2, 13, 17]. SINGH and SHINTON [18] have reported the usefulness of the measurement of this parameter in the diagnosis of pernicious anaemia. It, therefore, seems that the measurement of serum or plasma levels of pepsinogen can be used as an alternative, reliable and indirect, investigative tool for the diagnosis of gastric atrophy.

We have made an indirect attempt to assess the prevalence of pernicious anaemia in Delhi area in India using these parameters, i.e. serum pepsinogen, parietal cell antibody and serum B_{12} levels, and report our findings.

Material and Methods

Serum pepsinogen levels were measured by the method of EDWARDS *et al.* [11], which was subsequently modified [SINGH, unpubl. observations]. The serum and haemolysate of human red cells were incubated at pH 1.5–2.0 and the resulting free tyrosine-like substances were measured colorimetrically after precipitation of proteins. The values were finally expressed as units of pepsinogen per ml of serum.

Parietal cell antibody. The sera were examined for parietal cell antibody using freshly obtained gastric mucosa of rat as substrate and anti human γ-globulin fluorescent conjugate for delineating the antigen antibody complex on the substrate according to the technique described by IRVINE [14].

Serum vitamin B_{12} levels were determined in a microbiological assay system using Euglena gracilis [1]. By the use of this technique the serum levels were divided into a deficient range (less than 120 pg/ml), an intermediate range (120–180 pg/ml) and a normal range (over 180 pg/ml).

Haemoglobin levels were determined colorimetrically as cyanmethaemoglobin and thin films prepared from peripheral blood were stained with May-Grünwald-Giemsa stain and examined for morphological abnormalities.

Subjects Investigated. The study was carried out on 11 normal subjects, all males (referred to subsequently as 'control'), their age ranging from 25 to 42 years (mean 33 years). They were selected primarily on the basis that they all had haemoglobin levels above 13.0 g%, and they had not suffered at any time from rheumatoid arthritis, peptic ulcers and thyroid disorders. In addition, 87 patients

(60 males and 27 females) selected randomly from various out patient departments were also investigated These latter subjects had no demonstrable disorders as described above Their age ranged from 15 to 60 years (mean 35 4 years)

Results

Haematological findings Based on the haemoglobin levels the 87 patients selected from the out patient departments could be divided into 3 groups 23 subjects had haemoglobin levels of 13 0 g% or more, and were considered to be normal 43 subjects in whom haemoglobin level varied from 11 0 to 13 0 g% were considered to be an indeterminate group, for some of them were likely to be distinctly anaemic The remaining 21 subjects were anaemic and had haemoglobin levels between 4 5 and 10 9 g% The peripheral blood films from 47 subjects were examined for morphological abnormalities Of these, 17 showed predominantly hypochromic cells and 10 had numerous macrocytes but predom

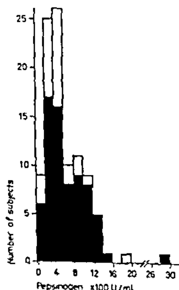


Fig 1 Distribution of serum pepsinogen in all subjects investigated Shaded columns indicate males and empty columns represent females

Table I Serum vitamin B_{12} in 82 subjects (11 control and 71 out patients)

Groups	Number of subjects	Serum vitamin B_{12} pg/ml	
		range	mean ± SD
Normal	47	180–480	316 ± 123
Intermediate	21	120–180	153 ± 19
Deficient	14	36–108	86 ± 20

inantly normochromic cells In the remaining 20 subjects the red cells were normocytic and normochromic

Parietal cell antibody The screening test for the parietal cell antibody, using anti human globulin fluorescent conjugate for delineating antigen antibody complex on the gastric mucosa of rats which were pre-incubated with patients' sera, gave negative results in all cases

Serum vitamin B_{12} This was measured in 82 subjects which comprised all 11 control subjects and 71 out-patients These findings, shown in table I, were divided in a deficient group (B_{12} level <120 pg/ml), an intermediate group (B_{12} level 120–180 pg/ml) and a normal group (B_{12} level >180 pg/ml)

Serum pepsinogen The serum levels of pepsinogen in all subjects considered together showed a positive skew distribution (fig 1) The levels in 71 males had a range of 0–2,825 U/ml (mean 617 U/ml, SD ± 447), and in 27 females ranged 55–2,000 U/ml (mean 520 U/ml, SD ± 373), the difference between the sexes was not significant ($p > 0\ 15$)

The distribution of serum pepsinogen levels in different age groups are shown in figure 2 and summarized in table II The highest levels of serum pepsinogen were observed in the age group 31–40 years, and these showed significant differences from the findings in the age groups 11–20 years ($p < 0\ 01$), 21–30 years ($p < 0\ 01$) and 41–50 years ($0\ 05 > p > 0\ 02$) The lowest levels observed in the age group 11–20 years also showed a significant difference ($0\ 10 > p > 0\ 05$) from the findings in the age group 21–30 years, but the difference from the findings in the age group 41–50 years was not significant ($p > 0\ 10$) The difference in distribution of pepsinogen levels in the age group 21–30 years (mean 537 U/ml ± 290) and 41–50 years (mean 507 U/ml ± 263) were not significant ($p > 0\ 45$) The serum pepsinogen levels in the age group 51–60 years (mean 700 U/ml ± 450) showed a significant difference from the findings in the age group 11–20 years ($p < 0\ 05$) But the differences be-

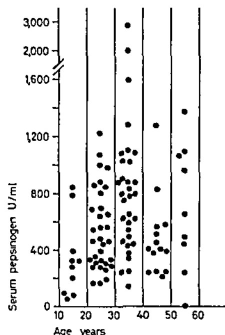
Table II Serum pepsinogen levels in the different age groups

Age groups years	Number	Serum pepsinogen, U/ml range	mean	SD
11-20	10	55- 481	352	288
21-30	33	156-1,218	537	290
31-40	32	147-2,825	861	526
41-50	14	222 1 275	507	263
51-60	9	0-1 087	700	450

tween the levels in the age group 51-60 years and those in the age groups 21-30 years ($p>0\ 10$), 31-40 years ($p>0\ 30$) and 41-50 years ($p>0\ 20$) were not significant

The serum pepsinogen levels in 11 control subjects had a range of 495-1,100 U/ml (mean 722 U/ml, SD ±447), which when compared to the findings amongst the 87 subjects from the hospital out patients (range 0-2,825 U/ml, mean 616, SD ±455) showed no significant difference ($p>0\ 40$) For further analyses the group comprising the hospital out patients was divided into 3 subgroups – (a) anaemic, (b) indeterminate, and (c) non anaemic – on the basis of haemoglobin levels as described in the earlier section The distribution of serum pepsinogen levels in these 3 subgroups are shown in comparison to those in the control group in figure 3 In 23 subjects in the non anaemic subgroup the serum pepsinogen levels range 55-1,370 (mean 560 U/ml, SD ±402) and showed no significant difference from the values obtained in the control group ($p>0\ 20$) In 43 subjects in the *indeterminate subgroup* in whom the haemoglobin levels fell between 11 0 and 12 9 g%, the serum pepsinogen levels (range 0-1,592, mean 608, SD ±427) did not have significant difference from the 'control' group ($p>0\ 30$) The anaemic subgroup, comprising 21 subjects with haemoglobin levels below 11 0 g%, the serum pepsinogen values (range 156-2 825, *mean 673 U/ml, SD* ±649) like the previous subgroups did not show significant difference ($p>0.50$) from the control group

The group of 47 subjects in whom the haemoglobin level was less than 12 0 g% were further sub-divided into (a) normocytic and (b) hypochromic subgroups, according to the morphological abnormalities of erythrocytes described in the earlier section, and the serum pepsinogen levels in these 3 subgroups were compared to each other As is shown in



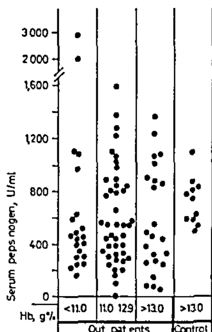
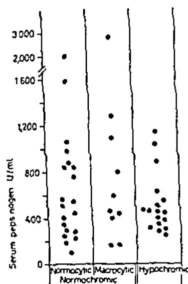


Fig 2 Distribution of serum pepsinogen in all subjects, according to their age

Fig 3 Distribution of serum pepsinogen in the 3 subgroups of out patient subjects shown in comparison with the 'control subjects

figure 4, the distribution of serum pepsinogen levels in these 3 subgroups were similar and the differences between their means were not significant

Relationship between serum levels of pepsinogen and vitamin B_{12}. For the purpose of this analysis 82 subjects (11 control and 71 out patients) on whom serum vitamin B_{12} levels had been assayed, were divided into (a) normal, (b) intermediate and (c) deficient groups, according to the criteria defined in the earlier section The serum pepsinogen levels in these 3 groups, determined according to their serum vitamin B_{12} levels, are shown in figure 5 The serum pepsinogen in the normal (range 101–2,825 U/ml, mean 699±455), and the intermediate (range 156–2,000, mean 796±469) groups were not significant ($p>0\,40$) The lowest levels of serum pepsinogen (range 239–784, mean 477±170) observed in the deficient group showed a significant difference ($p<0\,02$) from the findings in the intermediate group Similarly, the difference between the deficient and the normal groups were also significant ($0\,05>p>0\,02$) But the serum pepsinogen level in the normal and the intermediate groups were not different ($p>0\,40$)



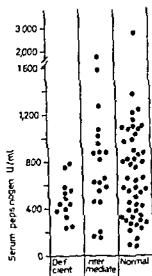


Fig 4 Comparison of serum pepsinogen in the subgroups based on red cell morphology 43 subjects were anaemic according to the criteria defined earlier In the remaining 4 Hb level had a range of 11 0–11 9 g%

Fig 5 Distribution of serum pepsinogen in 82 subjects (11 control and 71 out patient) divided into subgroups according to the serum vitamin B_{12} levels.

Discussion

The rarity of pernicious anaemia amongst Indians and other Asiatics is usually emphasized [5, 21] Several reports of well-documented cases of pernicious anaemia in these ethnic groups have appeared in recent years and it is obvious that the overt forms of this disease appear with greater frequency in this population than so far has been realized [3, 6, 9] but the prevalence of the latent form of the disease is not yet recognized

CALLENDER and DENBOROUGH [4] and MCINTYRE *et al* [16] found increased frequency of achlorhydria low levels of serum vitamin B_{12} and decreased urinary excretion of pepsinogen amongst close relatives of patients with pernicious anaemia and concluded that these were probably features of latent pernicious anaemia Subsequently, WANGEL *et al* [20] observed an association between gastric parietal cell antibody, low

serum vitamin B_{12} and pepsinogen levels amongst the first degree relatives of pernicious anaemia patients and recognized a higher frequency of prevalence of the latent form of this disease in these groups of subjects

During the present investigation the parietal cell antibody, serum pepsinogen and vitamin B_{12} levels were determined in an unselected population and the findings show a wide prevalence of some of the hallmarks of pernicious anaemia and suggest that, in the latent form, this disease is likely to be more commonplace than is so far recognized Parietal cell antibody was absent in all subjects studied which is in contrast with the findings of DESAI *et al* [10] who observed that the incidence of occurrence of parietal cell antibody was similar to that seen in the Western population These contradictory observations have no obvious explanation as the group of subjects included in the present study were ethnically identical to those comprising the study of DESAI *et al* [10] It is possible that this contradiction is the likely result of a different technique employed in this study

Low serum levels of pepsinogen and vitamin B_{12} were widely prevalent in the group of subjects investigated and the findings show that these two parameters had significant correlation in the same group of subjects It is now recognized that the low serum pepsinogen, an indirect evidence of reduced peptic cell mass and gastric atrophy, and low vitamin B_{12} levels are hallmarks of pernicious anaemia and even antedate the appearance of the anaemic phase of the disease It would, therefore, appear that the latent phase of pernicious anaemia has fairly frequent occurrence amongst the subjects included in this study Contrary to the findings of other workers [17–19], this study shows a characteristic distribution of serum pepsinogen, which in the age groups 11–20 and 41–50 years was significantly lower than in the other age groups under 50 years In addition, in these 2 age groups there is also a relatively higher concentration of serum pepsinogen levels below the lowest levels observed in the control subjects It would, therefore, seem that the age groups 11–20 and 41–50 years comprise a relatively higher proportion of subjects with reduced peptic cell mass There is some evidence that the parietal and peptic cells are relatively few at birth and develop subsequently [8] It is possible that the age group 11–20 years represents an intermediate stage in the course of full development of peptic cell mass It is also possible that the more commonly prevalent protein-calorie malnutrition in this age group is contributory to reduced peptic cell

mass, hence, lower serum pepsinogen, for varying degrees of gastric atrophy have been seen in monkeys subjected to prolonged protein-calorie deprivation [7]

The age group 41–50 years, consisting of a high proportion of subjects with low levels of serum pepsinogen, hence, reduced peptic cell mass, would be expected to include cases of true pernicious anaemia, as is known in the Western hemisphere It is of some significance that this age group contained a proportionately higher number of vitamin B_{12} deficient subjects It is also of some interest that the age group 41–50 years in this study, compared favourably with the age group in the Western hemisphere after which overt pernicious anaemia is diagnosed with increasing frequency, and is similar to the age which showed the peak incidence of this disease in the Western hemisphere during the early part of this century [5]

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Haemoglobin Bart's and Slow-Moving Haemoglobin X Components in Newborns

The Homozygous State for the Slow Moving X Components in a Malay Boy[1, 2]

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Abstract Newborns were examined for the presence of slow moving haemoglobin components tentatively designated X components and previously found in a group of Hb H disease in which invariably one of the parents of each patient had the same slow moving Hb X components also Structural studies showed that the abnormal haemoglobin in Chinese was identical with Hb Constant Spring an α-chain variant. Newborns with Hb Bart s and slow moving X components invariably had one parent with the X components also When the child grew older Hb Bart s disappeared while the Hb X components remained in the blood The homozygous state for the X components was found in a Malay boy through his newborn brother who had the X components in addition to Hb Bart s and had both parents with the X components One other Malay baby had the X components and Hb A_2 Indonesia inherited from the parents. The present study of newborns also showed that Hb Bart s can accompany different abnormalities of haemoglobin production, involving α-chains, β-chains as well as γ-chains Its presence in cord blood is, therefore, not specific for α thalassaemia

Key Words
Haemoglobinopathies
Hb Bart s
Slow moving Hb X
Thalassaemia

A recent study by LIE-INJO *et al* [6] revealed that haemoglobin H disease in Malaysia takes 2 forms, one accompanied by slow-moving components, tentatively designated X, and another without In those with X

[1] This work was supported by research grant HL 10486 and by the University of California International Center for Medical Research and Training (UC ICMRT) with Research Grant AI 10051 both from the National Institutes of Health, US Public Health Service

[2] Abstract of this paper was published in the proceedings of the XIVth International Congress of Haematology Sao Paulo, July 1972.

components, one parent invariably also had them, the other did not In those without the X components none of the relatives showed them Haemoglobin H disease with X components was then believed to be the result of a combination of the gene for X components with the gene for α-thalassaemia

Structural studies (in collaboration with Drs J B CLEGG and D J WEATHERALL) of the X components in a Chinese patient with Hb H disease in Kuala Lumpur showed that the abnormal haemoglobin is identical with Hb Constant Spring found in a Chinese family from Jamaica [2, 12] Because those in Malays and Indians may not be identical with those in Chinese and with Hb Constant Spring, the author temporarily retains the designation X components

A previous study of newborns in Malaysia [10] showed that newborns with Hb Bart's fell into 2 groups, one with more than 5% and the other with less than 3 2% of Hb Bart's This abnormality was then thought to represent 2 types of α-thalassaemia in the newborn period, although the possibility that it did not always do so was recognized No attempts were made at that time to look for the slow-moving Hb X components, which are clearly visible only in concentrated haemolysates After the presence of the X compounts in Hb H disease and their mode of inheritance became better understood, the author resumed the study of newborns, using more concentrated haemolysates in an attempt to find the X components in cord blood

The finding that the X components in one of our Chinese patients with Hb H disease were identical with Hb Constant Spring, an α-chain variant, stimulated the author even more to focus on newborns with Hb X components, realizing the importance of finding a homozygous case for such an α-chain variant because it would show directly whether 1 or 2 loci exist for the synthesis of α-chains of haemoglobin If a newborn and both parents were to have X components, then a sibling might be homozygous for this abnormality This paper presents the results of the study

Materials and Methods

Cord blood samples were collected randomly from babies delivered at the General Hospital Maternity Unit Kuala Lumpur Specimens were placed in acid citrate dextrose (ACD) solution and processed within 24 h of collection In addition 2 newborn babies were referred to us for consultation because of hydrops fetalis with unusual features

Haematological studies were carried out according to standard methods Concen
ted haemolysates were prepared in the usual way, except that slightly less than 1
of distilled water was added to the washed packed red blood cells and 0.5 vol
toluene

Haemoglobin was analyzed on starch gel electrophoresis with tris EDTA boric
d buffer at pH 8 0 and 8 6, discontinuous tris boric acid buffer at pH 9.5, and
osphate buffer at pH 7 7 Haemoglobin components were quantitated by the cel
ose acetate electrophoretic method of MARENGO-ROWE [11] and DEAE column
romatography of HUISMAN and DOZY [4] Alkali resistant haemoglobin was stud
l by the method of SINGER *et al* [15]

Results

Hb Bart's and the X Components in Healthy Newborns

In a study of 1,431 newborns of different racial groups (492 Malays, 01 Chinese and 438 Indians), 98 babies were found to have Hb Bart's in he cord blood Of these, 39 had Hb Bart's level above 5% and 58 had Hb Bart's level below 3 8%. And 1 had Hb Bart's level in between

No X components were found in babies with Hb Bart's level above % but these babies with appreciable amounts of Hb Bart's regularly howed 2 unusual components (fig 1) tentatively called Y components, one clearly seen at pH 8 6 (Hb Y_1) migrating between Hb F and Hb A_2 and the other clearly seen at pH 8 0 (Hb Y_2) between Hb Bart's and Hb A They usually accompany appreciable levels of Hb Bart's above 5% but a few of the newborns with levels lower than 5% had the Y components also However, the newborns with abnormal Hb X components that we found did not show them, at least not with the methods used They were described and discussed elsewhere by the author [5] Probably, they are the same as those seen in hydrops fetalis due to homozygous α-thalassaemia

Among babies with trace amounts of Hb Bart s, 7 (6 Malays and 1 Chinese) had Hb X components in addition to Hb Bart's the rest did not have them

Among those with Hb Bart s without X components, 2, both Indians had a slow-moving haemoglobin component tentatively designated Hb F Kuala Lumpur (Hb F KL) which moved faster than Hb A. and very slightly slower than Hb Y_1 Follow-up study in one, showed that the abnormal component disappeared when the child grew older A third Indian newborn with the same abnormal slow moving haemoglobin did not have any demonstrable amount of Hb Bart s The parents of all 3 with this abnormal haemoglobin did not show the abnormality, because it is a γ-chain

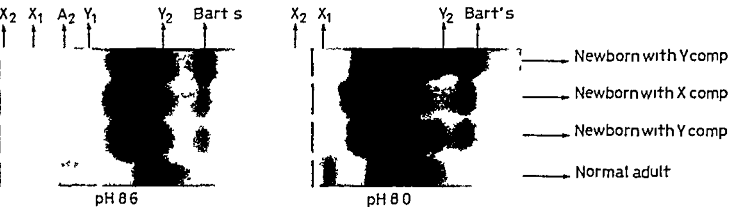


Fig 1 Starch gel electrophoresis, tris-EDTA boric acid buffer pH 8 6 and pH 8 0 showing Hb Bart s accompanying abnormal Hb production in the newborn No Y components are seen in those with X components Y_1 is clearly seen at pH 8 6, Y_2 at pH 8 0

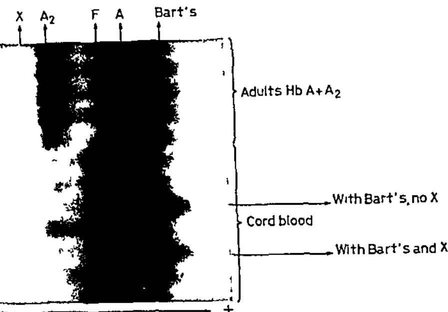


Fig 2 Starch gel electrophoresis in tris-EDTA boric acid buffer pH 8 6 showing a newborn with Hb Bart s without X component and another with Hb Bart s and X components

variant Structural studies on this abnormal haemoglobin are under way and a detailed report will be published elsewhere in collaboration with Dr B G WILTSHIRE and Dr H LEHMANN

12 babies had Hb E in the cord blood and one of the parents of each of them had also Hb E Four of these babies had Hb Bart's in addition to Hb E, 3 in trace amount and 1 in appreciable amount Follow-up study of one baby with Hb E and trace amount of Hb Bart's showed him to be a Hb E trait carrier Hb E in the newborn can easily be differentiated from increased Hb A_2 in discontinuous tris-boric acid buffer pH 9.5 At this pH the Hb E in the newborn moves faster than Hb A_2 It was also noticed that Hb E is usually associated with slow-moving haemoglobin components resembling the X components demonstrable after prolonged staining The significance of it is not known

37 babies with Hb Bart's without the X components (20 with Hb Bart s level above 5% and 17 with Hb Bart's level below 3 8%) were found to have parents also without the X components The babies with

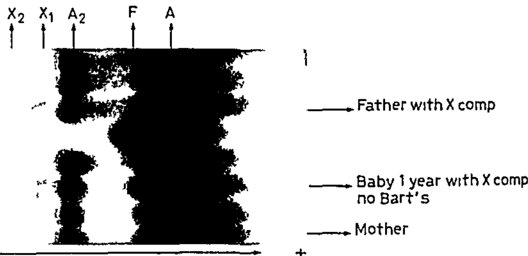


Fig 3 Starch gel electrophoresis tris EDTA boric acid buffer pH 8 6 showing the Hb pattern of a baby who had Hb Bart s and X components at birth At 1 year of age no Hb Bart s could be detected anymore, Hb X components are still present One of the parents has the X components

the X components invariably had one parent with the same abnormality and the other parent without, except for 1 who had both parents with the X components

Seven babies were found with the X components and trace amounts of Hb Bart's In these newborns the X_1 component sometimes had a slightly slower mobility at pH 8 6 than the X_1 component seen in the adult parent The X component amounted to an average of 0 6% of the total amount of haemoglobin No Y components could be detected in these babies with X components (fig 1) Four of the 7 newborns were re-examined when older

One Malay baby with Hb Bart's and X components (fig 2) who had a father with the X components and a mother entirely normal, was re-examined at the age of 1 year No Hb Bart's could be detected at this age while the X components were still clearly demonstrable (fig 3) Re-examination of both parents gave the same results as before

A second Malay baby with Hb Bart's and X components whose father had the X components and whose mother was normal, was re examined at the age of 5 months Again no Hb Bart's could be detected while the X components were still present and re examination of the parents gave the same results as before

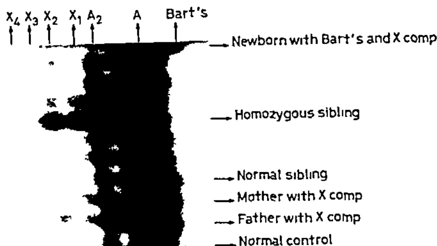


Fig 4 Starch gel electrophoresis discontinuous tris-boric acid buffer pH 9.5 showing newborn with Hb Bart s and X components Both parents had the X components one sibling was homozygous for the X components, one was normal and other siblings were trait carriers of X components.

A third Malay baby with Hb Bart s and X components had the father with X components and the mother with Hb A_2 Indonesia [8] At the age of 7 months the child no longer had Hb Bart s but still had the X components and also Hb A_2 Indonesia, apparently inherited from the mother which was, however, not demonstrable at birth This case will be reported separately in more detail

A fourth Malay baby had both parents with slow-moving X components Blood taken again 8 days after birth showed more or less the same pattern as that of his cord blood with Hb Bart s and X components (fig 4) Study of the whole family revealed 1 of 8 children a boy 12 years old, to be apparently homozygous for the X components He had hepatosplenomegaly and signs of haemolysis with mild haematological changes One other child was entirely normal and 6 others (including the newborn) were trait carriers of the abnormality (fig 4) The trait carriers had the same haemoglobin pattern as the parents with 2 slow moving components, the homozygote had 4 slow-moving haemoglobin components more clearly seen at pH 8 6 2 very pronounced and 2 much less pronounced (After prolonged staining in an 8 slot gel the shadow of 2 additional

slow-moving haemoglobin components in addition to the 2 usual ones could sometimes also be seen in the trait carriers) The major haemoglobin component in the homozygous person is, however, what seems to be Hb A, while a haemoglobin with the mobility of normal Hb A_2 was also demonstrable

Structural studies on the slow-moving X components are being carried out This case will be published in detail elsewhere

Hydrops and Erythroblastosis Fetalis with Hb Bart's and X Components

Two cases of mild hydrops fetalis not due to blood group incompatibility, one Chinese 36 weeks and one Aboriginal Malay 30 weeks old were found to have Hb Bart's between 3 3 and 3 8% and X components One was still born and the other died soon after birth One parent of each baby had the X components while the other had normal haemoglobin pattern The fetuses had hepatomegaly and ascites Peripheral blood changes with many nucleated red cells without sickling of the erythrocytes but with Hb H inclusion bodies were found Haemoglobin analysis showed in addition to Hb F and Hb A also a small amount of Hb Bart s and Hb X components and a shadow of Hb H Autopsy performed in one showed excessive extramedullary erythropoiesis in different organs These cases were mentioned in an earlier publication [6]

Discussion

Follow-up study of newborns with Hb Bart's and X components in the present series clearly showed that they had the X component trait because they were healthy and the Hb Bart's disappeared when they grew older leaving only the X components in the blood while one of the parents had the X components and the other was entirely normal Those with Hb Bart's higher than 5%, as well as those with Hb Bart's below 3 8% without the X components, did not have any parent with the X components Those with X components had one parent with the same abnormality Newborns with the X components or Hb F KL with Hb Bart's fell into the group with levels of Hb Bart's below 3 8% The number of babies with Hb X components is considerably smaller than the number of babies with Hb Bart's only without X components Compared with the frequencies of Hb X components in adults [7], the frequencies in newborn are much

lower Since not all parents of the 59 babies with trace amounts of Hb Bart's were examined, the possibility cannot be excluded that a few newborn trait carriers of Hb X components not showing the abnormal haemoglobin in cord blood, have been missed.

In the present series relatively many more newborns with trace amount of Hb Bart's were detected than in the previous study [10] probably due to the use of more concentrated haemolysates and a longer staining method

It is clear from the findings in this study that Hb Bart's in the newborn can be associated with a variety of abnormality of haemoglobin production, involving Hb Y_1 and Hb Y_2, Hb X components (α-chain variant), Hb F KL (γ-chain variant), Hb E (β-chain variant) and may be other unknown abnormalities, which await more sensitive techniques to be discovered Therefore, Hb Bart's in the newborn does not, or not always, represent α-thalassaemia and the concept of α $thal_1$ and α $thal_2$ in the newborn [16] apparently has to be corrected or at least modified.

The earlier assumption [6] that the X component gene may not be associated with Hb Bart's in the newborn period was incorrect. In fact, at that time we did find 2 cases of healthy newborns with X components and Hb Bart's These cases were, however, not discussed because we were not sure about their significance since the parents were at that time not yet examined We were particularly cautious because in newborns with Hb E, prolonged staining usually shows small slow moving components resembling X components even in fresh blood They are also seen in the parent with Hb E and in Hb E carriers in general, especially in the homozygous condition and in Hb E β thalassaemia Therefore, in this study, only after the parents had been examined and one parent of each baby had been found to have the X components without other abnormal haemoglobin were we convinced that they were in fact trait carriers of the X components

In the light of these findings, several questions raised in our earlier publication [6] can probably now be answered We wondered why NA-NAKORN *et al* [13] found that practically 100% of offspring of Hb H disease had Hb Bart's while we found part of Hb H disease cases to be the combination of the X component gene with α-thalassaemia. This is because carriers of the X components usually also carry Hb Bart's in the newborn period Probably, several newborns in the series of NA NAKORN *et al* [13] also had the X components, but were overlooked as happened in our earlier studies of α thalassaemia in newborns [10]

The 2 cases of hydrops and erythroblastosis fetalis with hepatomegaly described in this paper were mentioned earlier [6] They were then thought to represent Hb H disease in the newborn period, because of the severe clinical symptoms, the presence of many Hb H inclusion bodies in the red blood cells and the evidence of haemolysis not normally found in the trait condition of α-thalassaemia nor in that for Hb X components However, the exact genetic basis for this condition is still obscure They may represent the X component gene in combination with α thalassaemia More probably they are the combination of the X component gene with another genetic or non genetic factor Clearly, the term Hb H disease, until now used for all conditions associated with the presence of Hb H in the blood, even when acquired [1, 3, 14, 17], needs better delineation

The finding of a case of homzygous condition for the X components, with an abnormal haemoglobin pattern similar to that found in the trait carrier, but considerably more pronounced is most significant It shows again beyond doubt that the X components are a real expression of an abnormal gene and not a mere artefact If the abnormal haemoglobin in the Malay family proves to be Hb CS, an α-chain variant, this will provide direct evidence in the homozygous state for a duplication of α-chain locus, because this homozygous person for the α chain variant produces also what seem to be normal Hb A and normal Hb A_2 with normal α-chains in addition to the abnormal α-chains

Acknowledgements I like to thank the staff of the labour room of the Maternity Unit, General Hospital for their generous help in collecting blood samples for this study I am also grateful to my laboratory technicians for valuable technical help, especially to Mr ARNASALAM s/o Solai who, in addition to giving valuable technical help spent much of his free time in tracing relatives of newborns under study and in obtaining their co-operation

Addendum

Since completion of this study we have shown with the help of Dr J B CLEGG and Dr D J WEATHERALL that the slow-moving Hb X in the Malay family with the homozygous offspring is identical with Hb Constant Spring

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Also the paper by FOLAYAN ESAN [Brit. J Haemat. 22 73, 1972] has been published in which he came to the conclusion that Hb Bart s in the newborn Negro is not specific for α thalassaemia.

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The Failure of Normal Haemopoiesis in Rats during the Development of Acute Leukaemia

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Abstract The reaction of normal haemopoiesis under the development of a transferable acute leukaemia was studied in the rat A reduction of cell numbers was observed in the bone marrow in the proliferating compartments of erythropoiesis megakaryocytopoiesis and myelopoiesis In the peripheral blood, erythrocyte and platelet levels decreased to one tenth of normal Granulocytes and lymphocytes showed an increase coincident with the increase in circulating leukaemic cells before a final tendency to decline These changes are attributed partly to variations in the inflow from the bone marrow and partly to direct effects on the peripheral blood cells themselves The reduced number of cells in the proliferation compartment may be brought about by effects on this compartment itself, but there are some indications that a reduced inflow from the preceding stem cell compartment might be responsible

Key Words
Experimental leukaemia
Bone marrow failure
Leukaemia haemopoiesis
Rat bone marrow

In human acute leukaemia, anaemia, thrombocytopenia and granulocytopenia are common observations These symptoms are consequences of a failure of normal haemopoiesis The pathogenesis of this phenomenon in patients and its relation to the growth of the leukaemic cells remain, however, largely unknown Since the investigation of this problem in patients is still limited on methodological grounds and also because at the time when a diagnosis of acute leukaemia can be established the failure of normal haemopoiesis is already well-advanced, it is profitable to use an experimental animal model in which all 3 kinetic cell compartments (functional, proliferating or stem cell) can be investigated during the development of an acute leukaemia

In this paper, the haematology of an acute leukaemia in the rat is described, which has proved suitable for such an investigation by its con-

stant growth characteristics The change in numbers of normal haemopoietic cells during the development of this leukaemia is demonstrated and the possibilities of identifying in which cell kinetic compartment the defect may arise are discussed

Materials and Methods

Rats All investigations were carried out on rats of the inbred BD IX strain [6] Seven week-old rats of both sexes weighing 100–150 g were used

Leukaemia The leukaemia studied was the experimental rat leukaemia, L5222. It was originally chemically induced with ethylnitrosourea by IVANKOVIC [12] who found no evidence for virus infection (Gross technique) and classified it on cytochemical and morphological grounds as myelomonocytic This leukaemia is transferable by i.v or i.p injection of leukaemic cells. The rate of development is closely correlated with the number of cells transferred, and it is very reproducible in its course and its kinetics [9 10] Death of leukaemic rats occurs with a leukocyte count of about 400 000 cells/mm³ After transfer of 10^7 leukocytes, this cell number is reached on the 7th day [10]

Transfer Blood from a single leukaemic donor was sedimented with a Plasmagel® EDTA mixture and the supernatant leukocyte suspension diluted to the desired leukocyte concentration with physiological saline Transfer was made by i.v injection of 10^7 leukocytes in 0.5 ml to 36 recipients via a lateral tail vein. Six control animals received physiological saline only

Sacrifice The recipients were killed under ether anaesthesia in groups of 3 (at 12 and 144 h only 2 for technical reasons) at 12 hour intervals up to 6 days after the transfer of leukaemic cells Controls were sacrificed at the beginning and end of the experimental period [illegible] abdominal aorta just b [illegible] marrow were made for [illegible] for sectioning

Haematology Leukocyte and erythrocyte counts were performed in duplicate using a Coulter counter Platelets were counted microscopically by the method of BRECHER and CRONKITE [3] Blood and bone marrow smears were stained with May-Grünwald-Giemsa and the slides coded before evaluation In the peripheral blood smears, 200 nucleated cells and in bone marrow smears 1 000 nucleated cells were differentiated for each animal For the erythropoietic series 5 classes E_1–E_5 [5] and for the myelopoietic series 8 classes, M_1–M_8 [4] were identified. A separate differential count of 200 megakaryocytes, classified according to the description of EBBE *et al* [7] was made.

Bone marrow cellularity was determined from haematoxylin and eosin stained sections of tibial marrow by counting the number of nucleated cells in 50 random microscope fields using an eyepiece graticule, to a total area of 0.125 mm², including sinuses and blood vessels. Megakaryocytes were assayed in a separate count at a lower magnification to a total area of 2.5 mm² This enabled the d'f

ferential counts to be converted to absolute numbers of cells of each type per unit area of section

Results

The general pattern of increase of the leukaemic cell population in bone marrow and peripheral blood is presented in figure 1 The total number of nucleated cells in the bone marrow increases continuously up to about 10% above control values The leukaemic blast cells in the bone marrow multiply from the beginning and the results suggest an exponential growth throughout the period of observation At 6 days, the leukaemic cells constitute about 50% of the bone marrow population with a concomitant decrease in the normal haematopoietic cells to almost half the control values In the peripheral blood, leukaemic cells can first be identified 2 days after transfer From the 4th day onwards they increase precipitately and reach values of about 200,000 cells/mm³ at 6 days

The pattern of change in normal haemopoiesis is shown in figures 2–5, where the behaviour of the proliferating cells in the bone marrow and the corresponding functional cells in the blood are given In the *erythropoietic series* (fig 2) the basophilic normoblasts (E_2 and E_3) remain unaffected for 2 days and then decrease to one tenth of their initial value A fall in the polychromatic normoblasts occurs somewhat later In the late non-dividing normoblasts a surprising increase is seen after the first 2 days At this time they have very acidophilic cytoplasm and dense, pyknotic nuclei, suggesting that there is a defect in the normal extrusion of the nucleus and/or in the release of mature cells to the circulation In the peripheral blood, the erythrocytes remain constant for 3 5 days before falling by nearly 20% per day to about half their normal value

In contrast to the early erythropoietic proliferating cells, in the *myelopoietic series* (fig 3) the dividing stages (M_2–M_4) show no obvious changes until 4 5 days after transfer of leukaemia and then the decrease is only slight Metamyelocytes and juveniles decline moderately at about the same time as the dividing stages A decrease is seen in the number of marrow granulocytes over the whole period, which seems to be more rapid between the 2nd and 4th day, probably corresponding to the increase of the granulocytes in the peripheral blood

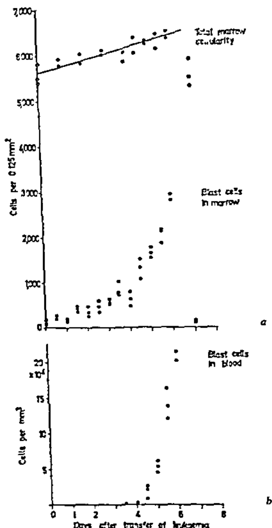


Fig. 1. *a* Increase in total marrow cellularity (upper curve) and leukaemic blast cells (lower curve) in the marrow with time after transfer of 10^7 leukaemic leukocytes. Both sets of data are presented as nucleated cells per unit area of tibial marrow section. The regression line shown for the total marrow cellularity was calculated by the method of least squares. *b* Increase of leukaemic blast cells in the peripheral blood. In all figures, the open circles represent control animals sacrificed at the beginning and end of the experiment (7th day)

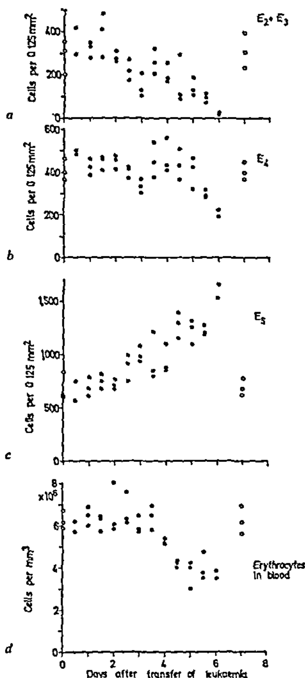


Fig 2 Changes in the numbers of erythropoietic cells *a* E_2 (large basophilic normoblast) and E_3 (small basophilic normoblast) The E_1 cells (pronormoblasts) have not been shown here because differentiation from leukaemic blast cells was not always certain *b* E_4 (*polychromatic normoblast*) *c* E_5 (*acidophilic normoblast* or late non dividing normoblast) *d* Erythrocytes in peripheral blood

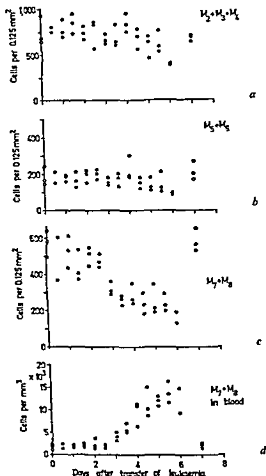


Fig 3 Changes in the numbers of myelopoietic cells. *a* Proliferating compartment in the bone marrow M_2 (promyelocytes) M_3 (immature myelocytes) and M_4 (mature myelocytes) The M_1 cells (myeloblasts) have not been shown here because of the uncertain differentiation from leukaemic blast cells. *b* Maturing compartment in the bone marrow M_5 (metamyelocytes) and M_6 (juveniles) *c* Functional compartment in the bone marrow M_7 (bands) and M_8 (segmented neutrophils) *d* Bands and segmented neutrophils in peripheral blood.

Megakaryocyte and platelet counts are shown in figure 4 No marked difference can be seen in the pattern of behaviour of the various megakaryocyte stages, probably owing to the small number of immature cells

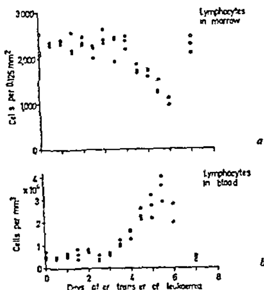


Fig 5 *a* Lymphocytes in the bone marrow *b* Lymphocytes in peripheral blood

stages of the leukaemia, megakaryocytes and platelets are reduced to one tenth of their normal values

The *change in number of the lymphocytes* (fig 5) in the bone marrow seems to be the reverse of that seen for the leukaemic blast cells In the peripheral blood, normal levels are maintained over 3 5 days followed by a 7- to 8 fold increase before a final tendency to decrease, as in the blood granulocytes

Discussion

The results of the experiment described here show clearly that during the development of the transferred leukaemia the recipient animals suffer anaemia and thrombocytopenia and in the final stages there is a tendency to granulocytopenia and lymphopenia There is also a marked reduction of normal haemopoiesis in the bone marrow and massive infiltration by leukaemic blast cells

The question arises as to whether any deduction can be made from these results concerning which cell kinetic compartment is affected by

the leukaemic cells In any compartment, an observed reduction in size can be brought about by a diminished input from the preceding compartment, by a shorter transit time or by direct damage to the cells themselves For the peripheral blood, it must therefore be considered to what extent the decrease of cell numbers in the peripheral blood is attributable to events in the bone marrow and to what extent it may be caused by factors in the circulation How far must the reduced haemopoiesis in the bone marrow be imputed to a reduced input from the stem cell compartment and how far could it be due to a shortened transit time or a direct action of LBC on proliferating cells?

In the erythropoietic system, the blood erythrocyte count falls much too quickly to be caused only by a reduced erythropoiesis in the bone marrow With an erythrocyte life span of about 50 days [1], even a complete cessation of erythropoietic cell production in the marrow would result in a fall in red cell count of only 2% per day Thus it must be concluded that the anemia seen is not occasioned by a reduced input from the bone marrow but is largely due to peripheral factors Measurement of plasma haemoglobin using benzidine have shown that haemolysis of circulating erythrocytes takes place That this haemolysis is mainly due to the leukaemic milieu is indicated by current studies with ^{51}Cr labelled cells, in which it has been found that erythrocytes from normal donors transfused to leukaemic recipients suffer the same rapid disappearance rate as the host's own erythrocytes

The relationship between blood granulocyte changes and myelopoiesis is not so clear The granulocytes in peripheral blood show an increase at the time when there is a release of large numbers of leukaemic blast cells into the circulation It is conceivable that the increase in granulocytes is to some extent due to a maturation of leukaemic blast cells along the granulocytic pathway, however, the growth of leukaemic blast cells in diffusion chambers has shown that although the cell number increases 10 fold there are no signs of maturation or differentiation of the cells Another explanation for the granulocyte increase could be the emptying of bone marrow reserves of granulocytes (M_7 and M_8) This suggestion is supported by the observation that the increase of blood granulocytes coincides with the decrease in M_7 and M_8 cells in the bone marrow In the final stages, there is a tendency for the blood granulocytes to decrease This decrease is of doubtful significance because of the limited period of observation but a similar tendency has been repeatedly observed in our earlier experiments with this leukaemia [11] and is

probably a consequence of the decrease in proliferating myelopoietic cells in the bone marrow

It is noteworthy that the lymphocytes in the peripheral blood increase half a day later than the granulocytes, and then show a similar final tendency to decrease This pattern has also been observed in earlier experiments [11] An explanation for this behaviour (perhaps loss of structure in bone marrow and lymph nodes) is not yet clear

There appears to be a good correlation between the thrombocytopenia and megakaryocyte decline in the bone marrow The random loss of rat platelets of 40% per day [8] requires a daily replacement of almost half the population This means that a change in the megakaryocytes in the marrow is quickly reflected in the platelet count in the blood. The decrease in platelet count observed here can be entirely attributed to the decrease in total megakaryocytes in the marrow which occurs about half a day earlier

The changes in the cell numbers in the peripheral blood are thus partly brought about by the decrease of normal haemopoiesis in the bone marrow and the consequent reduced input, but are also due to effects in the circulation As already discussed, reduction in size of the proliferating cell compartment could be due to diminished input from the stem cell compartment, to direct damage of the proliferating cells or to a shortened transit time through the bone marrow Regarding the last possibility, the magnitude of the changes observed, particularly in erythropoiesis and megakaryocytopoiesis, make it practically untenable. The effects of the other two possiblities cannot be satisfactorily separated in this experiment Nevertheless, some inferences can be drawn from the time course of the decrease in cell number in the various cell series during the development of the leukaemia Whereas the leukaemic blast cells grow exponentially in the bone marrow from the very outset, no change occurs in the normal cells until after 2 days, and then the different cell series begin to decline at different times If it is assumed that there is direct damage to proliferating cells, then it must be postulated that a certain minimum number of leukaemic cells are necessary (2–5% of the bone marrow population) and that each cell series has a different sensitivity to the leukaemic cells On the other hand, if there is a reduced inflow from a preceding stem cell compartment there could be a time lag before this makes an observable effect on the proliferating cells The different times of decrease in the various cell series might then be related to their varying proliferation rates or transit times through the

committed stem cell compartments For the recognizable proliferating cells, at least, there is evidence for differing bone marrow transit times, 2 days for erythropoiesis [2], 2–3 days for megakaryocytopoiesis [7], and 4 days for myelopoiesis [2]

The suggestion that the stem cell compartment is also involved in the failure of normal haemopoiesis under the influence of leukaemia is in agreement with experimental observations in mice [14] and with deductions from human acute leukaemia [13] Since the observations reported here have given indirect indications of stem cell involvement in the failure of normal haemopoiesis, more direct investigations of the resting bone morrow cells labelled with ^{3}H-thymidine by the continuous labelling method are in progress

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Interaction of Platelets with Ascitic Plasmacytoma *in vitro*[1]

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Abstract During interaction *in vitro* of syngenic platelets from healthy mice with HIPA ascitic plasmacytoma, clot formation was slower than during interaction of syngenic platelets from HIPA tumor bearing mice with HIPA plasmacytoma The platelet aggregates were composed, in both cases, of 'balloon platelets' and tumor cells The granulated part of the 'balloon platelets' made up the centre, whereas granule free spheroids were seen at the periphery of the aggregates To contact with platelets, the tumor cells reacted with formation of microvilli, condensation of mitochondria and phagocytosis of the granule-free spheroids. No formation of pseudopodia by platelets in contact with the tumor cells was observed

Key Words
'Balloon platelets'
HIPA plasmacytoma
Plasmacytoma of mice
Platelet phagocytosis

During the reaction of platelets with tumor cells *in vitro*, there was, according to WARREN [14], fibrin deposition, and formation and aggregation of so-called 'balloon platelets' 'Balloon platelets' were defined as platelets composed of two spheroids a small, granule-rich spheroid inside the aggregate and another spheroid, devoid of granules at the periphery Contact of syngenic platelets with tumor cells *in vitro* was established by pseudopodia-forming on the platelets

By contrast, we observed *in vivo* adherence of platelets to tumor cells of experimental HIPA plasmacytoma without the formation of pseudopodia [10] Presently, an *in vitro* system was used in order to investigate this discrepancy and to study the general behavior of syngenic platelets when mixed with HIPA plasmacytoma

[1] We are indebted to Mrs U WEHRLI for her excellent technical assistance

Material and Methods

HIPA Plasmacytoma The tumor used is an induced plasmacytoma of mice Its morphological and biological beatures have been previously reported [7-10 12]

adult BALB/c mice by cardiac puncture The blood was drawn into plastic syringes containing ca 15 IU/ml heparin The heparinized blood was centrifuged for 5 min at 1,500 rpm and the platelet rich plasma was drawn off for use within a short time of its preparation

Preparation of tumor-platelet mixtures Platelet rich plasma was drawn into syringes, each containing 0.5 ml HIPA plasmacytoma ascites obtained from the same mouse The tumor platelet mixtures were expelled into a heparinized clear plastic tube of 4 mm internal diameter and curved to form a circle of 12 cm diameter The tube was then attached to a plastic disc arranged at an angle of 60° and rotated at about 13 rpm As soon as a solid clot was noted the rotation was stopped and the time elapsed from the beginning of the rotation was recorded The material was then immediately prepared for electron microscopy

Electron microscopy The tumor platelet aggregates were fixed in 2% glutaric dialdehyde and treated as previously reported [10]

Experiments Two experiments were carried out Platelet rich plasma from HIPA plasmacytoma bearing BALB c mice (experiment 1) and from healthy BALB c mice (experiment 2) was mixed with HIPA tumor ascites from syngenic mice

Results

If HIPA tumor cells were mixed *in vitro* with platelets from HIPA plasmacytoma bearing mice (experiment 1) a solid white clot was noted in the plastic tube about 10 ± 2 min after the start of rotation Formation of the clot took about 20 ± 3 min if HIPA tumor cells were mixed with platelets from healthy BALB c mice (experiment 2) In electron micrographs of clots from both experiments numerous platelet aggregates were seen intermingled with tumor cells However, there were no morphological differences between platelet aggregates of experiment 1 and 2, respectively

The aggregates consisted of a core of fibrin with some free granules and so-called 'balloon platelets' of varying appearance Balloon platelets' were frequently undergoing medial division into 2 spheroids (fig 1-3, 5) This division yielded 2 morphologically different spheroids a granulated body with dense ground substance (body A) and a 'ghost



Fig 1 'Balloon platelet' aggregate between tumor cells Partial separation of the platelets in 2 bodies (arrows) A and B Adherence of platelet bodies, type B, to tumor cells is distinct No formation of pseudopodia CF = cytoplasmic fibrils in tumor cells × 15,000



Fig 2 Balloon platelet aggregate between tumor cells Complete separation of platelets in 2 bodies A and B Body type A granules and fibrin in center Granule free bodies, type B in contact with tumor cells. V = tumor cell villi × 15 000



Fig. 3. Invagination of body type B into tumor cell (arrow). F = fibrin. × 18,000.

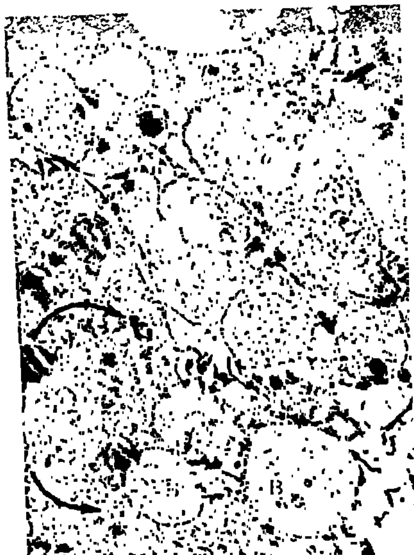


Fig. 4. Bodies, type B, incorporated in tumor cells. Aggregation of mitochondria nearby (curved arrows). Straight arrow pointing to microvilli. × 15,000

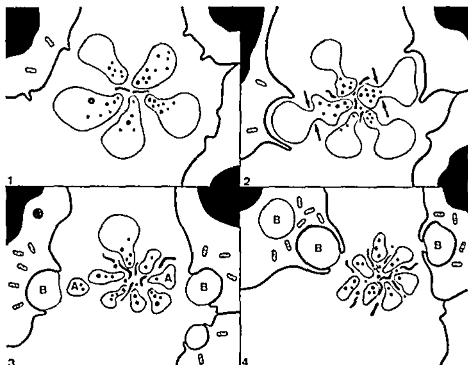


Fig 5 Diagram schematically illustrating interaction of platelets with HIPA tumor cells *1* Platelets aggregate between tumor cells The aggregate is composed of 'balloon platelets' *2* Separation of 'balloon platelets' by a process of medial separation *3* Body type A of 'balloon platelets' constitutes the center of the aggregate together with fibrin and some free granules Body type B in contact with tumor cells without formation of pseudopodia Tumor cells with microvilli and aggregation of mitochondria in region of contact *4* Phagocytosis of bodies type B by tumor cells

body' which was granule-free or poorly granulated with little ground substance (body B) Body type A, together with fibrin and free granules formed the central mass of the aggregates (fig 2, 5) Bodies of type B were usually located at the periphery of the platelet aggregates The membrane of both types of bodies, but especially of those of type B was frequently ruptured Contact of tumor cells with platelets or platelet bodies occurred through body B Platelets had no pseudopodia in relation to the points of contact (fig 1–4)

Tumor cells, i e undifferentiated plasmocytes, were usually well preserved *in vitro* (fig 1–4) Cytoplasmic fibrils were sometimes seen

(fig 1) Tumor cells reacted to contact with platelet bodies with formation of microvilli and aggregation of mitochondria in regions of contact (fig 2, 4, 5) Ghost bodies (B) appeared to be phagocyted by the tumor cells through a process of invagination (fig 3–5)

Discussion

Platelets originating from healthy BALB/c mice and from HIPA plasmacytoma bearing BALB/c mice, respectively, react similarly with HIPA tumor ascites *in vitro* However, clot formation is slower if platelets from normal mice are mixed with HIPA ascites The reason for this difference is not known, it seems, however, not related to the number of circulating platelets, since HIPA tumor-bearing mice are usually thrombocytopenic [12]

Platelet aggregates contain 'balloon platelets', fibrin and some extracellular granules The latter might originate from platelets by thrombocytorhexis, since 'balloon platelets' sometimes show disrupted membranes

In our experiments, balloon platelets' underwent medial division into 2 morphological different bodies The smaller granular bodies constitute the central mass of the aggregate Since the larger spheroids are free of granules and contain little ground substance, they probably represent the inactive part of the platelet The failure of formation of pseudopodia *in vitro* is in accordance with the *in vivo* findings in which platelets contacted HIPA tumor cells without formation of pseudopodia [10]

Inhibition of pseudopodia formation *in vitro* is probably a rather nonspecific phenomenon and may be due to different exogenous platelet-damaging agents, as platelet agglutinins or other factors [5, 11] In other cases, it may be caused by an inherent defect in platelets, as in thromboasthenia [1] The fact that platelets from healthy BALB/c mice fail to produce pseudopodia by contacting HIPA tumor cells *in vitro* supports the hypothesis that an external factor is responsible for the supposed deficiency Impairment of fibrin and platelet function in multiple myeloma has been frequently reported [4, 6, 13] PACHTER *et al* [6] described impaired release of platelet factor 3 in plasma from a macroglobulinemia patient, and using anti-macroglobulin serum tagged with fluorescein isocyanate adduced evidence for coating of platelets by macroglobulins. HIPA plasmacytoma does not presently produce detectable parapro-

teins [8, 9]; however, an abnormal protein may be present in HIPA ascites and disturb platelet function by a similar mechanism

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Acta Haemat 49 57–64 (1973)

In vivo Effect of Phytohaemagglutinin on Mouse Haemopoietic Tissue

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Abstract In vivo studies were made of the effect of a single (1 mg) dose of phytohaemagglutinin (PHA) on haemopoiesis in young female adult mice over a period of 20 days The results show that PHA stimulates erythroid cells to divide but produces many nuclear abnormalities It is suggested that these abnormalities may account for a poor reticulocyte response and the absence of any increase in the number of circulating erythrocytes An attempt is made to correlate these results with the results of PHA treatment of patients with aplastic anaemias.

Key Words
Bone marrow mitosis
Erythropoiesis
Haemopoiesis
Mouse bone marrow
Phytohaemagglutinin

Phytohaemagglutinin (PHA) stimulates lymphocytes in tissue culture to form blast like cells [12, 15, 16, 18, 21] Clinical studies by many workers on the effect of PHA on aplastic anaemias of various aetiologies have shown that PHA may produce remissions in some cases [1–4, 6, 8–11, 19] Analysis of these reports leads to the conclusion that drug induced aplastic anaemias respond better to PHA than do those of unknown aetiology Drug induced anaemias may, of course, recover spontaneously but it seems certain that PHA can stimulate or accelerate a remission Only 3 cases of aplastic anaemia of unknown origin seemed to be at all affected by PHA administration One of these [1] was improving before PHA treatment began and should be excluded One was affected only to the extent of showing a slight reticulocytosis (no information about the bone marrow was given) [11] and only one [2] showed any sig-

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nificant improvement. In many of the case reports of patients who responded to PHA, reticulocyte counts were not mentioned. In those in which they were, only 4 patients [1–4, 8] had a reticulocyte count of over 4%.

There appear to be few studies *in vivo* of the effect of PHA on the haemopoietic tissues of laboratory animals. ELVES *et al.* [5] gave weekly injections of PHA to rats for 4 weeks. They found no significant haematological change at the end of this time and the only histological change was a possible cuffing of large pale lymphocytes around the splenic follicles of some of the rats. GAMBLE [7] treated mice with PHA and found an increase in the weight of the spleen, accompanied by an increase in its total content of normoblasts, granulocytes and lymphoid cells.

The purpose of the experiments to be described was to study the effect of a single dose of PHA on the haemopoietic tissues and on reticulocyte and erythrocyte counts in healthy mice.

Material and Methods

20 C57BL female mice, each weighing 20–25 g, were given an i.p. injection of PHA (Burroughs Wellcome) 1 mg in 0.1 ml of pyrogen free water. Pairs of mice were sacrificed on alternate days starting on day 2 and ending on day 20. In addition 20 similar mice were each given an injection of 0.1 ml of pyrogen free water and a pair sacrificed as controls on alternate days. In order that the maximum number of metaphase figures should be seen in the tissue preparations, all the animals were given an i.p. injection of N-desacetyl-N-methyl colchicine (Colcemid, Ciba) 0.06 mg in 0.05% aqueous solution, 3 h before sacrifice [17]. Each animal was also given an i.p. injection of 100 IU of heparin in 0.1 ml of pyrogen free water 0.5 h before sacrifice in order to render its blood non-coagulable.

Under ether anaesthesia, each animal was exsanguinated by severing the great vessels at the root of the neck and the blood collected in a polythene beaker.

Erythrocyte and reticulocyte counts were performed on the blood. The erythrocytes were counted by eye in a counting chamber. The reticulocytes were stained with cresyl blue, the blood smeared on a slide and counted per 100 red cells at a magnification of 600 with an oil immersion objective. Smears of bone marrow were made by dispersing small amounts of femoral marrow on a slide in a drop of mouse serum and spreading. Smears were stained with MacNeal's tetrachrome stain at pH 5.4. Samples of spleen and liver were removed for histological section and fixed in Zenkers fluid. Sections 5 μm in thickness, were stained with haematoxylin and eosin.

At a magnification of 600, using an eye piece reticle, the number of metaphase plates in 5 000 nucleated cells in splenic red pulp and bone marrow were counted. Mitotic rates were expressed as metaphases per 100 nucleated cells. The liver, which

Demonstration of enzyme activities The water used was distilled and deionized; the acetone was redistilled. Before being incubated the tissue preparations were dipped in cold acetone for 5 minutes to remove lipids and then in 2 changes of chilled 0.1 M phosphate saline, pH 7.5, for a total of 5 minutes to remove water soluble endogenous substrates.

a) Some sections were then incubated to demonstrate the location and degree of activity of Δ^5-3β hydroxysteroid dehydrogenase, an enzyme occurring early in the pathway for steroid hormone biosynthesis (*Samuels* 1960; *Rubin et al.* 19[illegible]; *Deane & Rubin* 1965). The medium is given in Table 9. The control medium was similar except that it lacked DHA. Generally 4 to [illegible] coverslips were placed in a Coplin staining jar and incubations were carried out for 1 h and 3 h at 40 °C.

Table 9

Composition of medium for demonstrating the activity of Δ^5-3β hydroxysteroid dehydrogenase

	Amount
Isotonic saline buffered with 0.1 M phosphate, pH 7.5	4.0 ml
Nitro blue tetrazolium (NBT, Sigma) 1 mg/ml H_2O	2.0 ml
Dehydroepiandrosterone (DHA, Sigma) 0.5 mg/ml acetone	0.5 ml
β-Nicotinamide adenine dinucleotide (NAD or DPN, Boehringer) 6 mg/ml H_2O	1.0 ml

b) For demonstrating the activity of NADH-tetrazolium reductase, otherwise known as NAD-diaphorase, a total of 7.5 ml of medium contained buffer saline and [illegible] above, plus 5 mg $NADH_2$ (or DPNH). Incubations usually ran for [illegible]

c) For [illegible] reductase activity [illegible]

genera[illegible]

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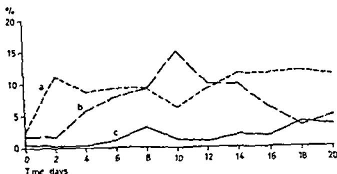


Fig 1 Single dose PHA a = Bone marrow mitotic rate per 100 nucleated cells b = Splenic red pulp mitotic rate per 100 nucleated cells c = Reticulocytes in peripheral blood per 100 erythrocytes against time in days

in normal adult mice is not haemopoietic [20] was examined for areas of haemopoiesis.

Results

Figure 1 is a graph of reticulocyte count and mitotic rate of splenic red pulp and bone marrow against time The counts in the controls did not alter throughout the course of the experiment

Peripheral blood Erythrocyte counts in the experimental animals did not differ significantly from the controls The reticulocyte counts in the PHA treated animals showed a gradual increase to over 4 times the normal value by the 18th day

Bone marrow There was an increase in the mitotic rate on the 2nd day of 3 5 times the control value and this was maintained throughout the time of the experiment The apparent fall on day 10 (fig 1) is not statistically significant ($p = 0.2-0.1$)

Only 55–60% of the cells in mitosis were obvious erythroblasts Most of the others were leucocyte precursors Many of the metaphases in both the myeloid and erythroid lines were abnormal during the first few days of the experiment There appeared to be 2 types of abnormality The commoner was that of 2 metaphase plates in 1 cell with in many cases, an obvious attempt to divide into 2 daughter cells Figures 2 and 3 illustrate 2 such cells which have almost completed this division The other

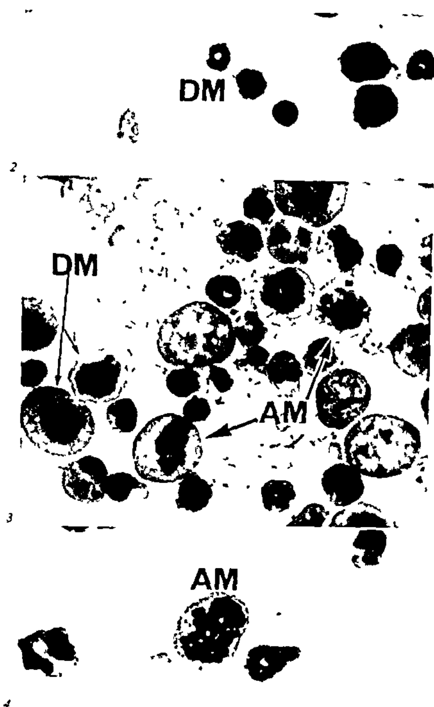


Fig 2 Two metaphase plates in a single cell labelled (DM) from the marrow of a mouse treated with PHA and arrested with Colcemid

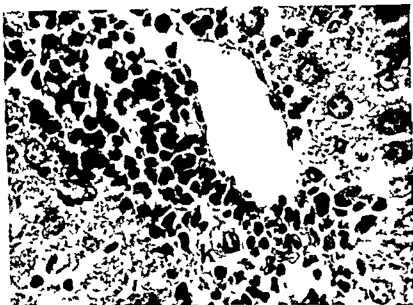


Fig 5 Portal area from the liver of a mouse treated with PHA and mitosis arrested with Colcemid There are numerous erythroblasts in mitosis and a megakaryocyte labelled (k) can be seen

type of abnormality is a variable degree of disarray of the chromosomes Examples of this are seen in figures 3 and 4

Splenic red pulp Most of the cells in mitosis were of the erythroid series There was a gradual rise in mitotic rate lasting from the 4th to the 14th day with a peak at the 10th day, after which it decreased gradually to normal levels

Liver A large number of erythroblasts in mitosis were seen in aggregations near the veins of the portal system Many were also scattered singly throughout the parenchyma Figure 5 shows a number of these dividing cells and also a megakaryocyte No evidence of haemopoiesis could be

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Fig 3 Two metaphase plates in a single cell labelled (DM) and 2 cells labelled (AM) showing disarray of the chromosomes. Mouse bone marrow treated with PHA and mitoses arrested with Colcemid

Fig 4 Cell labelled (AM) showing disarray of the chromosomes Bone marrow of mouse treated with PHA and mitoses arrested with Colcemid

seen in the livers of the control animals All parameters in the control animals remained unchanged throughout the experiment

Discussion

Normal erythropoiesis can only take place in the presence of certain factors Some of these, such as vitamin B_{12} and folic acid are known, but it is probable that there are others, as yet unknown, which are also needed Many of the patients with aplastic anaemias who were treated with PHA showed an active bone marrow but little or no haematological improvement These patients may have been lacking some factor needed in the later stages of maturation In the experiments described there was a great increase in erythroblastic activity in bone marrow, splenic red pulp and liver in mice treated with PHA yet only a slight reticulocytosis and no increase in erythrocyte count Even if the patients who showed an increase in marrow activity but not in erythrocyte count were lacking some factor needed later in maturation, there is no reason to suppose that the normal mice used in these experiments were deprived of any such factor

It is necessary, therefore, to seek some other explanation for the failure of PHA to produce an increase in erythrocyte count in mice Some of the possibilities are as follows (1) The animals were not anaemic and there was no call for more erythrocytes so most of the reticulocytes may have remained in the place where they were formed This would not explain the lack of response in anaemic patients (2) Cells may be lost during maturation [13] The large number of abnormal mitotic figures seen in the experiments described may have indicated death of the cell (3) If reticulocytes are formed they may be abnormal in some way, as many of their precursors are, and rapidly destroyed by the reticulo-endothelial system Lozzio [14] reported that large doses of PHA caused a fall in erythrocyte count, a decrease in red cell survival time and an increased take-up by the spleen of ^{51}Cr-labelled cells It is possible that PHA also stimulates the cells of the reticulo-endothelial system and that whether there is an increase in circulating red cells depends on a balance between red cell production and destruction

In conclusion, the results of these experiments show that PHA stimulates mitosis of cells in all the haemopoietic centres in mice but that it produces many nuclear abnormalities and no increase in circulating erythrocytes It is difficult to understand the significance of these results It is

possible that the dose of PHA is important in determining whether the response is normal or abnormal mitosis and whether there will be a consequent increase in circulating cells

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Factors Influencing the Uptake of Vitamin B_{12} by Normoblastic and Vitamin B_{12}-Deficient Bone Marrow Cells

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Abstract The uptake of serum bound ^{57}Co-B_{12} by freshly aspirated human bone marrow cells is an active, calcium-dependent process requiring the presence of cellular respiration, oxidative phosphorylation and free sulphydryl groups In contrast, serum mediated uptake of ^{57}Co-B_{12} by reticulocytes and erythrocytes has been reported to be an energy independent, surface adsorption phenomenon. Bone marrow cells also take up ^{57}Co-B_{12} in the absence of serum Such uptake is dependent on cellular respiration but not on calcium ions or free sulphydryl groups. The average value for the uptake of serum bound ^{57}Co-B_{12} by vitamin B_{12}-deficient bone marrow cells was 2.7 times greater than that for normoblastic marrow cells

Key Words
Bone marrow cells
Metabolic inhibitors
Vitamin B_{12} deficiency
Vitamin B_{12} uptake

Several studies have shown that a protein present in normal serum promotes the uptake of vitamin B_{12} by mammalian cells [1, 2, 4, 5, 6] In the majority of these investigations, tumour cells or non-dividing cells such as human reticulocytes have been used. The characteristics of vitamin B_{12} uptake by rapidly dividing, non malignant cells such as haemopoietic cells have not yet been adequately studied In this paper we present data on the factors influencing vitamin B_{12} uptake by cells from normoblastic and vitamin B_{12}-deficient megaloblastic marrow aspirates. In addition, the recent observation that cell suspensions prepared from vitamin B_{12}-deficient human bone marrow take up more radioactive vitamin B_{12} than cells from normoblastic marrow [8] has been investigated in a larger number of patients.

Materials and Methods

Freshly aspirated bone marrow was mixed with 5 ml Hanks solution containing preservative free heparin The marrow aspirate was forced through a 21 gauge needle once and a 25 gauge needle twice and the resulting cell suspension centrifuged at 1 100 g for 5 min The buffy coat was separated washed 3 times in Hanks solution and finally resuspended in 4–8 ml of Hanks' solution The nucleated cell count and red cell count were determined using a Coulter counter (Model S)

^{57}Co labelled cyanocobalamin (^{57}Co-B_{12} 15–60 μCi/μg) was obtained from the Radiochemical Centre Amersham Uptake of ^{57}Co B_{12} was determined using a modification of the method of RETIEF *et al* [6] To 1 ml of the marrow cell suspension was added 1 ml Hanks solution 2 ml of 0 9% NaCl containing 10 mM $CaCl_2$ and 1 ml of autologous serum pre-incubated with 1 ng ^{57}Co-B_{12} The use of Hanks solution instead of NaCl $CaCl_2$ was later shown not to affect uptake 1 ng of ^{57}Co B_{12} per ml serum has been shown to be a suitable subsaturating dose in previous studies [6 8] The mixture was incubated at 37 °C in a shaking water bath After the appropriate time interval the cells were washed 3 times in ice cold 0 9% saline and lysed in 2 ml of distilled water The uptake of ^{57}Co-B_{12} was measured by counting the lysate in a Packard scintillation counter In the majority of the experiments each culture bottle contained 0 8–2 0 × 10^7 nucleated cells and less than 0.5 × 10^9 erythrocytes Duplicate cultures were frequently set up using 1 ml Hanks solution or 1 ml 0 9% NaCl instead of autologous serum Uptake of ^{57}Co-B_{12} under these conditions is described as saline mediated uptake in this paper

As marrow suspensions contain many more mature erythrocytes than nucleated marrow cells, the contribution of erythrocyte uptake to the total uptake in marrow cultures had to be estimated For this purpose 4–6 different dilutions of washed red cells were prepared from each of 6 healthy individuals and the serum mediated uptake by 1 ml of each red cell suspension determined The effect of 5 mM 2 iodoacetamide on red cell uptake was also studied

The effects of temperature and a number of chemicals on the uptake of ^{57}Co B_{12} by bone marrow cells were also investigated To study the influence of various metabolic poisons sufficient Hanks solution was added to 1 ml of the marrow suspension to make a total volume of 4 ml after the addition of the chemical under study The pH of this mixture was adjusted to 7 4 when necessary Marrow cells were pre incubated with the metabolic inhibitor for 15–30 min at 37 °C before the addition of 1 ml serum containing 1 ng ^{57}Co-B_{12} and further incubation at 37 °C for 60–90 min

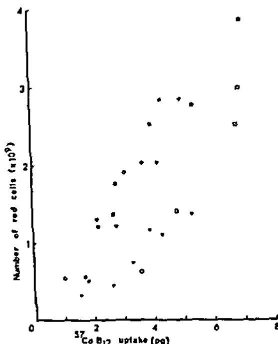


Fig 1 Serum-mediated uptake of ^{57}Co-B_{12} by varying numbers of normal erythrocytes

pared from venous blood and contained less than 1.5% of reticulocytes It is evident that there is a linear relationship between ^{57}Co-B_{12} uptake and erythrocyte numbers, and that 10^9 red cells may take up to 4 2 pg (range 1 7–4 2 pg) of vitamin B_{12} The average uptake by 0.5×10^9 red cells calculated from these data was 1 7 pg The transfer of serum bound ^{57}Co-B_{12} to erythrocytes was not inhibited by 5 mM iodoacetamide

Figure 2 shows the uptake of ^{57}Co-B_{12} by cell suspensions from 15 normoblastic and 9 vitamin B_{12}-deficient marrows, after 90 min incubation at 37 °C. Cell suspensions prepared from normoblastic marrows contained an average of 0.36×10^9 erythrocytes per 10^7 nucleated cells and those prepared from B_{12}-deficient marrows contained $0 21 \times 10^9$ erythrocytes per 10^7 nucleated cells The average value for serum mediated uptake by cells from normoblastic marrows was 3 1 pg per 10^7 nucleated cells and the corresponding value for B_{12}-deficient marrows, 8 4 pg per 10^7 nucleated cells There is some overlap between the two

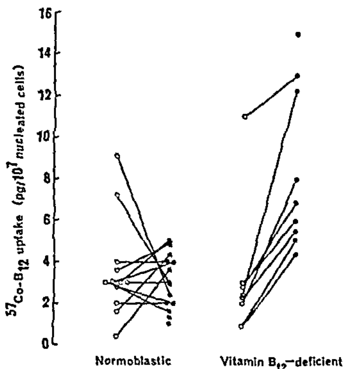


Fig 2 Uptake of ^{57}Co B_{12} by marrow suspensions ○ = Saline mediated uptake, ● = serum mediated uptake Lines connect results for the same marrow

groups, caused by the inclusion of two mildly megaloblastic marrow aspirates in the study

Saline-mediated uptake by cells from normoblastic marrows varied widely, being higher than, equal to, or less than serum mediated uptake Saline mediated uptake was always less than serum mediated uptake in the cultures from vitamin B_{12}-deficient marrow aspirates The average value for saline-mediated uptake was 3 5 pg per 10^7 nucleated cells in the normoblastic group and 3.2 pg in the vitamin B_{12}-deficient group The ratio between *serum-mediated and saline-mediated uptake* was 0 9 in the normoblastic group and 2 6 in the megaloblastic group

Figure 3 shows the rate of uptake of ^{57}Co-B_{12} by marrow cells over a 45–90 min incubation period Transfer of serum-bound ^{57}Co-B_{12} to marrow cells was initially very rapid and gradually diminished thereafter Serum-mediated uptake at 90 min was 1 8–2 4 times the value at 5 min in normoblastic cultures and 1 5–2 7 times that at 5 min in vitamin B_{12}-deficient cultures

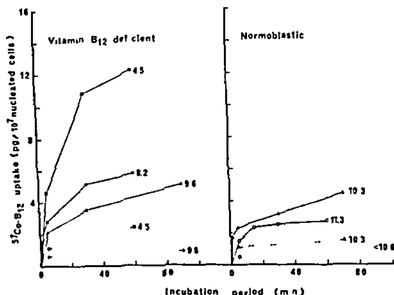


Fig 3 Rate of uptake of $^{57}Co-B_{12}$ by vitamin B_{12}-deficient and normoblastic marrow suspensions. ——— = Serum-mediated uptake, ---- = saline-mediated uptake The numbers opposite each curve are haemoglobin values in g/100 ml.

The effect of temperature on serum mediated uptake was similar for normoblastic and vitamin B_{12}-deficient marrows. Uptake of $^{57}Co-B_{12}$ at 4 °C was 30% lower than that at 37 °C, after a 20 min incubation period

Table I shows the effects of various metabolic inhibitors on the serum mediated transfer of $^{57}Co-B_{12}$ to marrow cells The results were similar in cultures from normoblastic and vitamin B_{12}-deficient marrows and are, therefore, not given separately The inhibition of uptake seen with disodium ethylenediamine tetra acetate (Na_2EDTA) sodium cyanide, 2 iodoacetamide and 2,4-dinitrophenol does not result from cell death caused by these metabolic inhibitors as dead bone marrow cells take up more vitamin B_{12} than living cells. Thus, marrow cells killed by fixation in absolute methanol for 10 min took up approximately 4–5 times more serum bound $^{57}Co-B_{12}$ than living cells. Serum mediated uptake of $^{57}Co-B_{12}$ was increased to a similar extent in the presence of 10% formalin

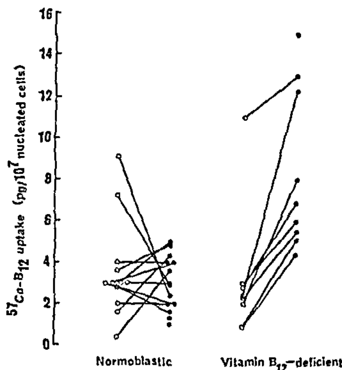


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Saline-mediated uptake by cells from normoblastic marrows varied widely, being higher than, equal to, or less than serum-mediated uptake. Saline-mediated uptake was always less than serum-mediated uptake in the cultures from vitamin B_{12}-deficient marrow aspirates. The average value for saline-mediated uptake was 3.5 pg per 10^7 nucleated cells in the normoblastic group and 3.2 pg in the vitamin B_{12}-deficient group. The ratio between serum-mediated and saline-mediated uptake was 0.9 in the normoblastic group and 2.6 in the megaloblastic group.

Figure 3 shows the rate of uptake of ^{57}Co-B_{12} by marrow cells over a 45–90 min incubation period. Transfer of serum-bound ^{57}Co-B_{12} to marrow cells was initially very rapid and gradually diminished thereafter. Serum-mediated uptake at 90 min was 1.8–2.4 times the value at 5 min in normoblastic cultures and 1.5–2.7 times that at 5 min in vitamin B_{12}-deficient cultures.

erythrocytes. The contribution of erythrocyte uptake to the total uptake in marrow cultures would, therefore, be similar for both normoblastic and vitamin B_{12}-deficient marrow cultures.

The earlier finding of higher serum-mediated uptake by vitamin B_{12}-deficient marrow cultures, compared to normoblastic cultures [8], has been confirmed in a larger number of patients. However, patients with mildly megaloblastic marrows and haemoglobin levels within or near the normal range, either showed only slightly increased serum mediated vitamin B_{12} uptake, or uptake within the normoblastic range. The average value for saline mediated uptake was similar in the normoblastic and vitamin B_{12}-deficient groups.

Serum mediated transfer of ^{57}Co B_{12} to cells from normoblastic and vitamin B_{12}-deficient marrows showed several similarities. Uptake was progressive over a 90-min period, sensitive to changes in temperature, and was markedly inhibited by 10^{-3} M Na_2EDTA. Inhibition by Na_2 EDTAwas corrected by the addition of 1.5×10^{-3} M calcium chloride, indicating that uptake was dependent on the presence of calcium ions. The transfer of ^{57}Co-B_{12} to marrow cells was appreciably depressed in the presence of 50 mM sodium cyanide, indicating that uptake was dependent upon cellular respiration. Iodoacetamide (5 mM) also caused significant inhibition, presumably due to combination with sulphydryl groups [5] of a hitherto unidentified enzyme or intracellular transport protein which is necessary for normal uptake. It is unlikely that the latter effect is due to inhibition of glycolysis, as two other inhibitors of glycolysis, sodium fluoride and sodium arsenate, were without effect.

PARANCHYCH and COOPER [5] found that uptake by Ehrlich ascites cells was only partly inhibited by 0.1 mM 2.4-dinitrophenol (an inhibitor of oxidative phosphorylation) and concluded that ATP was not directly involved in vitamin B_{12} uptake. In the present study, the uptake of serum bound ^{57}Co-B_{12} by bone marrow cells was significantly, although only slightly, affected in the presence of 5 mM dinitrophenol. In contrast the uptake of serum bound ^{57}Co-B_{12} by HeLa cells was strongly inhibited by 5 mM dinitrophenol [3]. These differences probably reflect variations in metabolic activity in different cell types. For example, ATP requirements and intracellular ATP stores may vary considerably in these three cell types leading to different rates of depletion of ATP when oxidative phosphorylation is uncoupled.

The transfer of serum bound vitamin B_{12} to bone marrow cells (normoblastic or vitamin B_{12}-deficient) is, therefore, an active, energy-de-

Table I Effect of metabolic inhibitors on serum bound ^{57}Co-B_{12} uptake by bone marrow cells (normoblastic and vitamin B_{12}-deficient)

Concentration M	Inhibitor	Inhibition[1] %
5×10^{-2}	NaCN	39 2
5×10^{-3}	2,4-dinitrophenol	16 1
5×10^{-3}	2 iodoacetamide	37 9
10^{-2}	Na_2EDTA	78 4
10^{-2} / $1\,5 \times 10^{-2}$	Na_2EDTA + $CaCl_2$	0 0
10^{-3}	NaF	1 0
10^{-3}	$NaHAsO_4$	0 0

[1] Each value represents the mean of 6–10 experiments

Saline-mediated uptake of ^{57}Co-B_{12} by bone marrow cells was unaffected by Na_2EDTA, 2-iodoacetamide and 2,4 dinitrophenol but was depressed by 44 9% in the presence of 50 mM sodium cyanide

Discussion

The average value for serum mediated uptake by normoblastic marrow cultures incubated for 90 min at 37 °C was 3 1 pg ^{57}Co B_{12} per 10^7 nucleated marrow cells The data on normal erythrocytes indicate an average uptake of 1 7 pg per 0 5 × 10^9 red cells As an average of 0 36 × 10^9 erythrocytes were present per 10^7 nucleated marrow cells, not more than 50% of the 3 1-pg uptake could be attributed to uptake by red cells That the larger number of erythrocytes does not mask uptake by 10^7 nucleated marrow cells is supported by the finding of progressively increasing uptake in normoblastic marrow cultures, over a 90-min incubation period Whereas most of the uptake by erythrocytes occurs within the first 5 min of incubation [6], uptake by normoblastic marrows was nearly twice as much at 90 min as at 5 min

Preliminary studies indicate that the serum-mediated uptake of ^{57}Co-B_{12} by macrocytes is similar to that by normal erythrocytes [unpublished observations] Thus, reticulocyte-poor red cell suspensions from 5 vitamin B_{12}-deficient patients showed an uptake of 2 1–3 5 pg/10^9

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pendent process, differing from vitamin B_{12} uptake by human reticulocytes (and erythrocytes) which appears to be largely an energy-independent surface adsorption phenomenon [6] As vitamin B_{12} is required for DNA synthesis during cell proliferation [7], anucleate reticulocytes would be expected to have little requirement for it No significant differences between marrow cells and tumor cells were detected [3, 5]

Two phases have been distinguished in the uptake of ^{57}Co-B_{12} by Ehrlich ascites cells an energy-independent primary uptake phase which is complete in less than 1 min, followed by an energy-dependent secondary uptake phase [5] The primary uptake phase was considered to represent a physico-chemical adsorption of serum bound cyanocobalamin to receptors at the cell surface We made no attempt to distinguish between primary and secondary uptake in marrow cultures, as primary uptake by the small number of nucleated cells would have been obscured by that due to the larger number of erythrocytes Marrow suspensions virtually free of mature erythrocytes will be required for such studies Nevertheless, the observation that iodoacetamide does not inhibit serum-mediated uptake by erythrocytes whereas it does depress uptake by bone marrow cells suggests that primary uptake is not dependent on the presence of free sulphydryl groups

Saline-mediated uptake of ^{57}Co-B_{12} by bone marrow cells was partially inhibited by 50 mM sodium cyanide, indicating a dependence on cellular respiration However, unlike serum mediated uptake, saline-mediated uptake was not dependent on calcium ions or free sulphydryl groups Evidently, two different mechanisms are involved in the uptake of serum-bound and free ^{57}Co-B_{12} As vitamin B_{12} is normally bound to specific transport proteins in the serum, it is likely that only serum-mediated uptake is of physiological importance Saline-mediated uptake is primarily a laboratory artefact but may be operative *in vivo* when there is an excess of unbound vitamin B_{12}

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he higher susceptibility to form Heinz bodies in the circulation of the newborn infant, possibly by alterations of the red cell membrane phospholipids

Since we only found a slightly increased Heinz body formation by acetylphenylhydrazine in adult erythrocytes circulating in the newborn infant 24 to 36 h after exchange transfusion, we reinvestigated this problem both *in vivo* and *in vitro*

Methods

The Heinz body formation of erythrocytes by acetylphenylhydrazine was determined in cord blood of normal newborn infants and in blood obtained from adults for the purpose of exchange transfusion Heinz body formation was further measured before and after exchange transfusion in cord blood of 3 infants with anti D erythroblastosis, 2 infants with ABO erythroblastosis and in 1 infant with unconjugated hyperbilirubinemia without blood group incompatibility Exchange transfusions were performed with 330 ml of fresh blood/kg body weight anticoagulated with 2,000 IU of preservative free heparin/l blood to which 25 000 U penicillin G/100 ml had been added

In vitro incubations All blood samples were anticoagulated by the addition of 20 IU of preservative free heparin ml blood After separation of erythrocytes from plasma by centrifugation (4 °C, 2 000 *g*) the cells were washed 3 times in isotonic saline Red cells of newborn infants were resuspended in the plasma of adults with identical blood groups and vice versa. For control, erythrocytes of newborn infants and adults were suspended in their own plasma The erythrocyte suspensions were then incubated under sterile conditions in an air atmosphere at 37 °C for 20 h and slightly agitated (300 oscillations/h) The incubations were performed in the presence of 300 mg glucose/100 ml suspension and when indicated with 100 mg calciumgluconate/100 ml

Heinz body formation The formation of Heinz bodies was measured before and after exchange transfusion and during *in vitro* incubations Erythrocytes were washed twice with isotonic saline 3 ml of packed red cells were resuspended in 6 ml of a buffered medium consisting of 3 parts of isotonic sal ne and 1 part of 0 12 M Sørensen phosphate buffer pH 7 4 Acetylphenylhydrazine and glucose were added to the medium giving final concentrations in the suspension of 7.5 and 11 0 mMoles/l respectively During incubation at 37 °C, aliquots of the suspensions were removed in intervals of 30 min for counting of Heinz body containing erythrocytes after staining the cells with Nilbluesulfate The results were expressed as the number of Heinz body-containing cells per 100 erythrocytes, counting 1 000 cells per preparat on in duplicate

Results

The high susceptibility of cord blood erythrocytes to acetylphenylhydrazine is documented in table I Three infants with anti D erythro-

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Heinz Body Susceptibility of Red Cells and Exchange Transfusion[1]

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Department of Pediatrics, University of Hamburg, Hamburg

Abstract Using blood which was anticoagulated with heparin the Heinz body susceptibility against acetyl phenylhydrazine of adult donor erythrocytes increased only slightly in the circulation of newborn infants within 30 h after exchange transfusion The high susceptibility of the donor erythrocytes found by KLEIHAUER *et al* using ACD for anticoagulation 12 h after exchange transfusion might be due to the effect of Ca^{++} given during exchange transfusion During *in vitro* incubations, the addition of Ca^{++} increased the Heinz body susceptibility of adult erythrocytes to the level known from newborn infants

Key Words
Erythrocytes of newborn
Heinz bodies
Exchange transfusion

The high susceptibility of cord blood erythrocytes against Heinz body-producing drugs and the higher incidence of Heinz body anemia in premature newborn infants are well documented [1, 3] However, extensive studies of the red cell metabolism failed to explain conclusively why cord blood erythrocytes produce Heinz bodies in the presence of oxidants to a greater extent than do erythrocytes of adults both *in vivo* and *in vitro* [review see 4] Moreover, ULUKUTLU *et al* [6] using the hemoglobin elution technic clearly demonstrated that Heinz body formation of cord blood erythrocytes does not depend on their high hemoglobin F concentration Recently, KLEIHAUER *et al* [2] showed that Heinz body production in erythrocytes of adults by acetylphenylhydrazine within 12 h following exchange transfusion in the newborn infant nearly increases to the level known from infants before exchange transfusion The authors concluded that the adult donor erythrocyte might acquire

[1] This work was supported by the 'Deutsche Forschungsgemeinschaft', Bad Godesberg Germany, grants Schr 86/8 and Schr 86/9

the higher susceptibility to form Heinz bodies in the circulation of the newborn infant, possibly by alterations of the red cell membrane phospholipids

Since we only found a slightly increased Heinz body formation by acetylphenylhydrazine in adult erythrocytes circulating in the newborn infant 24 to 36 h after exchange transfusion, we reinvestigated this problem both *in vivo* and *in vitro*

Methods

The Heinz body formation of erythrocytes by acetylphenylhydrazine was determined in cord blood of normal newborn infants and in blood obtained from adults for the purpose of exchange transfusion Heinz body formation was further measured before and after exchange transfusion in cord blood of 3 infants with anti D erythroblastosis, 2 infants with ABO erythroblastosis and in 1 infant with unconjugated hyperbilirubinemia without blood group incompatibility Exchange transfusions were performed with 330 ml of fresh blood/kg body weight anticoagulated with 2,000 IU of preservative free heparin/l blood to which 25 000 U penicillin G/100 ml had been added

In vitro incubations All blood samples were anticoagulated by the addition of 20 IU of preservative free heparin/ml blood. After separation of erythrocytes from plasma by centrifugation (4 °C, 2,000 g) the cells were washed 3 times in isotonic saline Red cells of newborn infants were resuspended in the plasma of adults with identical blood groups and vice versa. For control erythrocytes of newborn infants and adults were suspended in their own plasma. The erythrocyte suspensions were then incubated under sterile conditions in an air atmosphere at 37 °C for 20 h and slightly agitated (300 oscillations/h) The incubations were performed in the presence of 300 mg glucose/100 ml suspension and when indicated with 100 mg calciumgluconate/100 ml

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Results

The high susceptibility of cord blood erythrocytes to acetylphenylhydrazine is documented in table I Three infants with anti D erythro-

Table I Heinz body formation before and after exchange transfusion

	Incubation with acetylphenylhydrazine, h		
	0	1	2
	Heinz body-containing erythrocytes, %±SD		
Cord blood erythrocytes before exchange transfusion (n=6)	0	55.0±4.8	100
Adult blood donors (n=6)	0	0	10.2±2.4
Adult erythrocytes 30 h after exchange transfusion (n=6)	0	0	23.2±3.8

blastosis, 2 infants with ABO erythroblastosis, and 1 infant with unconjugated hyperbilirubinemia without incompatibility, in whom exchange transfusions were necessary were combined in one group. There were no significant differences concerning Heinz body formation among the infants of this group. Erythrocytes of adult blood donors showed a much slower and smaller Heinz body formation than cord blood erythrocytes. The difference between the 2 groups is highly significant ($p<0.001$). The erythrocytes of the blood donors were again studied after circulation in the newborn infants for 30 h. Heinz body susceptibility was higher than before exchange transfusion ($p<0.005$), but far below the values reported by KLEIHAUER *et al.* [2] 12 h after exchange transfusion. This increase may be due to heparin, whereas the anticoagulant ACD did not alterate Heinz body susceptibility neither in erythrocytes of adults nor in those of newborn infants.

To prove the hypothesis whether or not adult erythrocytes aquire the higher susceptibility in the plasma of newborn infants adult red cells were incubated in plasma of newborn infants under slight agitation *in vitro*. Figure 1 shows that Heinz body susceptibility is only slightly higher than the susceptibility of cells which were incubated after identical handling in their own plasma. The lower part of the figure demonstrates the higher susceptibility of cord blood erythrocytes both after incubation in their own plasma and in the plasma of adult donors. The addition of 100 mg of calcium gluconate/100 ml of incubation mixture increases Heinz body formation only minimally in cord blood erythrocytes, but markedly in adult erythrocytes both after incubation in their own plasma

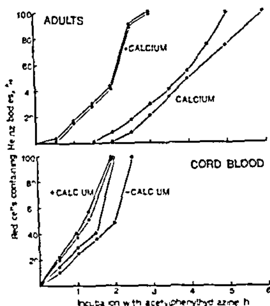


Fig. 1. Effect of calciumgluconate on the susceptibility of cord blood and adult erythrocytes during *in vitro* incubation. Adult and cord blood erythrocytes have been incubated for 20 h in their own plasma (●), adult erythrocytes in the plasma of cord blood (○) and cord blood erythrocytes in the plasma of adults (○) with and without the addition of calciumgluconate. At the end of the incubation the plasma has been removed as described under 'Methods'. Then the Heinz body formation has been determined in a second incubation in the presence of acetylphenylhydrazine. The graph represents a typical experiment (n = 6)

and after incubation in the plasma of newborn infants (upper part of fig. 1). The extent of Heinz body formation is now nearly identical with that of cord blood erythrocytes with and without the addition of calciumgluconate.

Discussion

As recently described by KLEIHAUER *et al.* [2] we also found an increase of Heinz body susceptibility of adult erythrocytes in the circulation of the newborn infant. However, in our studies, Heinz body formation was only one tenth of that observed by KLEIHAUER *et al.* who had

used ACD for anticoagulation of donor blood Since exchange transfusions in our Department are performed with fresh heparinized blood we need no calcium gluconate during exchange procedure Therefore, it is conceivable that the addition of calciumgluconate may accelerate the rate of Heinz body formation during exchange transfusion Our *in vitro* studies give further emphasis to this hypothesis The addition of calcium gluconate in a quantity similar to that used for exchange transfusions performed with ACD blood to the incubation mixtures increases Heinz body formation of adult erythrocytes by acetylphenylhydrazine to the levels observed in cord blood erythrocytes

Further studies may explain the effect of calcium on adult erythrocytes We found that isolated erythrocyte membranes (ghosts) of cord blood erythrocytes prepared with hypotonic phosphate buffer contain significantly more hemoglobin than the ghosts of adult erythrocytes prepared under indentical conditions Preincubation of adult blood with calciumgluconate increases the hemoglobin content of the ghosts This increase correlates well with their Heinz body susceptibility [5] Therefore, we suggest that membranes of cord blood erythrocytes primarily contain more hemoglobin which is denaturated predominantly in the form of Heinz bodies by acetylphenylhydrazine In adult erythrocytes, on the other hand membranes contain less hemoglobin and, therefore, exhibit a much lesser pronounced Heinz body susceptibility Calcium may increase the sol to gel transformation of the membrane proteins as described by WEED *et al* [7] and secondarily, the hemoglobin content of the membranes which is followed by an increased susceptibility to acetylphenylhydrazine

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Acta Haemat 49 80–88 (1973)

Immunzytologische Untersuchungen über das Verteilungsmuster von Ig-Determinanten bei Myelompatienten

Hinweise für eine klassenspezifische Restriktion auf B-Zellebene

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Abstract In 3 patients with myeloma and paraprotein containing cells in the peripheral blood, we demonstrated by an immunofluorescent technique an increase in the number of lymphocytes and plasma cells with immunoglobulin determinants of paraprotein class specificity Lymphocytes found with mebranous and intracytoplasmic paraprotein content were considered as intermediate forms in plasma cell development Some possible disturbances of cell proliferation known in lymphoproliferative diseases are discussed

Key Words
Immunofluorescence
Immunoglobulins
Lymphocytes
Paraproteins
Plasma cells
Plasmacytoma
Waldenstrom's disease

Untersuchungen an monoklonalen Zellproliferationen erleichtern das Verständnis der normalen Zelldifferenzierungsvorgange, da genugend einheitliches Material zur Verfugung steht und damit die Verhaltnisse übersichtlicher werden So zeigten immunzytologische Untersuchungen an Patienten mit monoklonalen Paraproteinamien, dass nicht nur morphologisch eindeutig als Plasmazellen identifizierbare periphere Blutzellen das Paraprotein intrazytoplasmatisch enthalten, sondern auch periphere Lymphozyten aller Grossenordnungen [2, 3, 9] Diese Beobachtung legte eine Differenzierung von Plasmazellen aus Lymphozyten auch unter normalen Bedingungen nahe, wofur neuere kombinierte autoradiographisch-elektronenoptische Studien weitere Argumente liefern [4]

Zahlreiche Untersuchungen sprechen dafur, dass knochenmarksabhangige B-Zellen Vorstufen Antikorper produzierender Zellen sind [1, 6, 10, 13, 18, 20] Sie lassen sich durch Immunglobulin(Ig)-Determinanten an ihrer Oberflache charakterisieren, die selbst wieder spezifisch Ig bzw Antigen binden und damit Zellproliferation und Antikorperbildung

induzieren können. Obwohl gesichert ist, dass Plasmazellen ganz überwiegend nur jeweils eine Ig-Klasse synthetisieren und sezernieren können [16], sind die Zusammenhänge zwischen Ig-Determinantenspezifität der B-Lymphozyten und sezernierter Ig-Klasse der Plasmazellen noch wenig untersucht. Immunzytologische Beobachtungen an Patienten mit Plasmazelleukämie legen nahe, dass schon auf B-Zellebene eine gewisse restriktive Determinierung erfolgt ist.

Material und Methodik

Patienten

1. 9 Normalpersonen.

2. 42jähriger Patient (R. A.) mit Plasmazelleukämie vom IgG λ-Typ. Von 7600/mm³ peripheren Leukozyten wurden 34% Lymphozyten und 12% Plasmazellen differenziert. Im Sternalmark fanden sich vermehrt Plasmazellen und lymphoide Retikulumzellen. GE 9,3 g%. Mancini: IgG zirka 10000 mg%, IgA 30 mg%, IgM 30 mg%. Esbach 7‰. Keine Knochenherde.

3. 74jähriger Patient (St. J.) mit terminaler Plasmazelleukämie bei IgA κ Plasmozytom. Von 5800 Leukozyten/mm³ waren 43% Lymphozyten und 2% Plasmazellen im Differentialblutbild. Im Sternalmark waren ganz überwiegend Plasmazellen. GE 9,3 g%. Mancini: IgG 1920 mg%, IgA 628 mg%, IgM 3040 mg%.

4. 68jähriger Patient (F. B.) mit Lymphosarkomatose und Paraproteinämie vom IgM λ-Typ. 6800 Leukozyten/mm³ mit 11,5% Lymphozyten und 3,5% Plasmazellen. GE 9,3 g. Mancini: IgG 1920 mg%, IgA 628 mg%, IgM 3040 mg%.

5. Drei Myelompatienten mit unauffälligem peripherem Blutbild (IgG κ, IgG λ, IgA λ).

Immunzytologische Untersuchungen

Der intrazytoplasmatische Ig-Gehalt wurde an methanolfixierten Ausstrichen peripherer Leukozytenkonzentrate bestimmt [2, 9].

Zum Nachweis von Ig-Determinanten wurden jeweils 4 × 10⁶ säulengetrennte, periphere Lymphozyten mit 0,05 ml Antiserum vom Kaninchen 20 min bei Zimmertemperatur inkubiert, dann in isotoner Hanks-Lösung gewaschen und anschliessend mit fluoreszierendem Antikaninchenserum versetzt (Sandwichmethode). Es wurden Antihumanseren (anti κ [illegible]) vom Kaninchen und ein an FITC gekoppeltes Antikaninchenserum von der Ziege der Fa. Behringwerke, Marburg/L., verwendet, zusätzlich ein selbst hergestelltes Antihuman-IgG-Serum.

Die Qualität der einzelnen kommerziellen Chargen war sehr unterschiedlich, nur ein Teil der angebotenen Proben war für diesen Nachweis genug hochtitrig. Die Monospezifität der Antiseren wurde immunelektrophoretisch und an fixierten Knochenmarkausstrichen von Myelompatienten getestet. Ein Tropfen einer insgesamt 9mal gewaschenen, homogenen Lymphozytensuspension wurde dann auf einen Objektträger aufgebracht und mit einem Deckgläschen versehen. Bei 400facher Vergrösserung wurden zunächst die Zellzahl im Hellfeld, anschliessend jeweils die

fluoreszierenden Zellen desselben Gesichtsfeldes im UV Licht ermittelt Pro Ansatz wurden mindestens 100 Zellen ausgezählt [weitere Methoden siehe 1, 11]

Ergebnisse

Bei 3 von 6 Patienten mit Paraproteinämien wurden immunzytologisch *in peripheren Leukozytenkonzentraten* vermehrt mononukleare Zellen mit intrazytoplasmatischem Paraproteingehalt nachgewiesen Während Normalpersonen und Myelompatienten ohne leukämisches Blutbild im peripheren Blut etwa 1‰ Ig haltige Zellen aufwiesen, waren bei diesen 3 Patienten 3,16 bzw 60% periphere mononukleare Zellen positiv (Abb 1 und 2) Der Vergleich mit dem Differentialblutbild zeigte, dass auch ein Teil der panotpisch als Lymphozyten differenzierten Zellen (Abb 3) paraproteinhaltig war Das Verteilungsmuster von Ig-

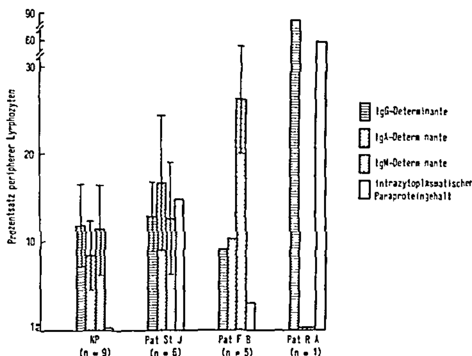


Abb 1 Verteilung der Ig Determinanten peripherer lymphoider Zellen bei Normalpersonen und 3 Patienten mit Ausschwemmung von paraproteinhaltigen Zellen Prozentueller Anteil von peripheren Blutzellen mit IgG IgA und IgM Determinanten n = Anzahl der Personen oder Einzelbestimmungen

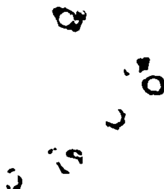


Abb 2 Intrazytoplasmatischer IgG Nachweis in peripheren lymphoiden Zellen des Patienten R A mit Plasmazelleukämie vom IgG λ Typ × 400 Ein analoger Prozentsatz von Zellen war an Parallelausstrichen λ positiv

Oberflächendeterminanten mit γ-, α-, und μ-Spezifität war bei Normalpersonen 12,0±4,9, beziehungsweise 8,2±4,0 und 11,3±5,7 Eine ähnliche Verteilung fand sich auch bei den 3 Myelompatienten (2 vom IgG-κ-Typ, 1 vom IgA-κ-Typ) ohne vermehrte Ausschwemmung paraproteinhaltiger Zellen

Demgegenüber waren beim Patienten R. A mit Plasmazelleukämie vom IgG-λ-Typ, dem Patienten St J. mit einem IgA-κ-Myelom und terminaler Plasmazellausschwemmung und dem Patienten F. B mit Makroglobulinamie eine deutliche Zunahme peripherer lymphoider Zellen mit Ig Determinanten der Paraproteinqualitat zu beobachten (Abb 4) Soweit diese Methode morphologische Aussagen zulasst, hatten neben Lymphozyten auch Plasmazellen Ig Determinanten Dies fiel besonders bei den beiden Patienten mit Plasmazelleukamie auf, wahrend beim Patienten mit Morbus Waldenstrom vorwiegend Lymphozyten μ-positiv waren

Rein rechnerisch hatte ein Teil der Lymphozyten von den Patienten St J und R A Ig-Determinanten und intrazytoplasmatisch Ig der Paraproteinqualitat Unter zytostatischer Therapie kam es beim Patienten R A zu einem gleichsinnigen Ruckgang von lymphoiden Zellen mit Membran- und intrazytoplasmatischem Ig wahrend Plasmazellen deutlich langsamer reagierten Zu einem späteren Zeitpunkt, im Stadium der Teilremission, waren nur mehr, etwa 1%, periphere mononukleäre Zel-

fluoreszierenden Zellen desselben Gesichtsfeldes im UV Licht ermittelt Pro Ansatz wurden mindestens 100 Zellen ausgezählt [weitere Methoden siehe 1, 11]

Ergebnisse

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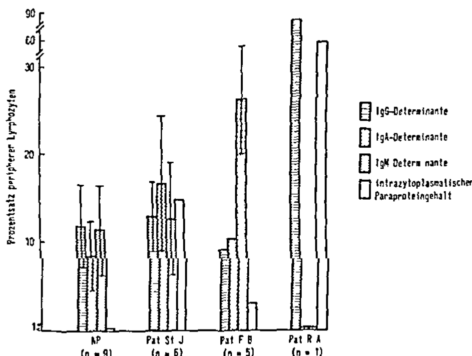


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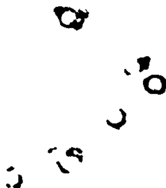


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Demgegenuber waren beim Patienten R A mit Plasmazelleukämie vom IgG λ-Typ, dem Patienten St J mit einem IgA-κ-Myelom und terminaler Plasmazellausschwemmung und dem Patienten F B mit Makroglobulinamie eine deutliche Zunahme peripherer lymphoider Zellen mit Ig Determinanten der Paraproteinqualität zu beobachten (Abb 4) Soweit diese Methode morphologische Aussagen zulasst, hatten neben Lymphozyten auch Plasmazellen Ig Determinanten Dies fiel besonders bei den beiden Patienten mit Plasmazelleukamie auf, wahrend beim Patienten mit Morbus Waldenstrom vorwiegend Lymphozyten μ positiv waren

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Abb 3. Panoptische Färbung eines peripheren Blutausstriches des Patienten R A mit atypischen, lymphoiden Zellen × 600

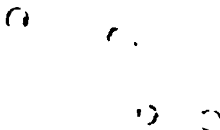


Abb 4 Zellsuspension gereinigter, peripherer Lymphozyten mit positiven μ-Determinanten des Patienten St J × 400 Sichelförmige, spezifische Fluoreszenz *nach Inkubation mit Antihuman-μ-Serum vom Kaninchen und anschliessender* Darstellung durch fluoreszierendes Antikaninchenserum

len – überwiegend Plasmazellen – paraproteinhaltig, etwa 4% mononukleärer Zellen hatten γ-Determinanten

Diskussion

In den B Lymphozyten sieht man heute aufgrund zahlreicher tierexperimenteller Ergebnisse die Vorstufen Ig produzierender Plasmazellen [1, 10, 12, 13, 16, 20, 21] Ungeklärt ist allerdings bisher noch, ob die für Plasmazellen postulierte Restriktion auf die Synthese von nur einer Ig-Klasse [16 u a] auch schon für B-Lymphozyten gilt bzw ob die Ig-Oberflächendeterminanten der Lymphozyten der von den Plasmazellen sezernierten Ig Klasse entsprechen Untersuchungen mit der Immunzytoadhäsionsmethode sprechen dafür, dass eine gewisse Pluripotenz der B Lymphozyten insofern noch gegeben ist, als etwa $^{1}/_{2}$ der Mausemilzzellen gleichzeitig 7S γ_1- und 7S γ_2-Oberflächendeterminanten aufweisen [12] und etwa 5% der peripheren, humanen Lymphozyten Rezeptoren für κ- und λ-Ketten besitzen [5, 8] Auch bei einigen Patienten mit chronischer Lymphadenose wurden Lymphozyten mit μ- und γ-Determinanten beschrieben [14]

Patienten mit Paraproteinämien scheinen besonders zum Studium dieser Frage geeignet sofern Intermediärformen der monoklonalen Zellproliferation ins periphere Blut ausgeschwemmt werden Dies ist vor allem bei Patienten mit Morbus Waldenström und Plasmazelleukämien der Fall [2, 19] Die immunzytologische Untersuchung von peripheren Leukozytenkonzentraten zeigt, dass in diesen Fällen bis zu 60% der peripheren Lymphozyten und lymphoidzelligen Formen paraproteinhaltig sind Vergleichende elektronenmikroskopisch immunzytologische Studien beweisen dass ein dem immunzytologischen Nachweis ähnlicher Prozentsatz peripherer lymphoider Zellen die für die Eiweisssynthese erforderlichen Zellorganellen besitzen und somit auch tatsächlich zur Proteinsynthese in der Lage sind [3] Einheitlich wurde eine korrespondierende Vermehrung von Lymphozyten mit Oberflächendeterminanten der Paraproteinqualität bisher lediglich bei Patienten mit M Waldenström bzw Makroglobulinämie beschrieben [19] Auch bei unserem Patienten F B wurden vermehrt Lymphozyten mit μ-Determinanten gefunden Das Verteilungsmuster der Ig Determinanten von B Zellen bei Patienten mit Plasmazelleukämien wurde bisher noch nicht untersucht Unsere Ergebnisse zeigen dass auch bei diesen Paraproteinämieformen im peripheren

Blut vermehrt lymphoide Zellen mit den korrespondierenden Ig-Determinanten gefunden werden und zumindest ein Teil der Plasmazellen noch Ig-Determinanten besitzen. Bei Patienten mit Paraproteinämien ohne Ausschwemmung von paraproteinhaltigen Zellen war hingegen die Verteilung der Ig-Determinanten peripherer Lymphozyten unauffällig, so dass Adsorbtionsphänomene ausgeschlossen werden konnten. Die Verteilung von Lymphozyten mit Ig-Determinanten und intrazytoplasmatischem Ig Gehalt bei den beiden Patienten mit Plasmazelleukämie legt nahe, dass ein Teil der Leukozyten nach beiden Kriterien positiv ist. Unter Zellkulturbedingungen konnte kürzlich gezeigt werden, dass je nach Differenzierungsgrad unterschiedlich viel Membran-Ig und intrazytoplasmatisches Ig gebildet wird und dass die Synthese dieser beider Ig-Formen getrennten Steuermechanismen unterliegen [13]. Es dürfte sich demnach bei jenen Zellen um die lichtoptisch nicht fassbaren Intermediärformen im Rahmen der Plasmazellentwicklung handeln.

Unsere Ergebnisse sprechen dafür, dass bei Patienten mit M. Waldenström und Plasmazelleukämien zumindest teilweise eine Restriktion der Ig-Determinantenspezifität bereits stattgefunden hat. Auch bei Patienten mit chronischer lymphatischer Leukämie handelt es sich in den meisten Fällen um eine Lymphozytenproliferation mit γ-μ-Determinanten, bzw. um eine monoklonale B Zellproliferation [7, 11, 17]. Zum Unterschied von den vorgenannten lymphoproliferativen Erkrankungen scheint jedoch bei Patienten mit Lymphadenose ein Block in der Weiterdifferenzierung zu Plasmazellen zu bestehen, da Plasmazellen gegenüber der Norm vermindert sind [11, 19] und in den Spätstadien der Erkrankung häufig Antikörpermangelzustände auftreten.

Zusammenfassung

Bei 3 Myelompatienten mit Ausschwemmung von paraproteinhaltigen Zellen in das periphere Blut liess sich immunzytologisch eine Vermehrung von Lymphozyten und Plasmazellen mit Ig Determinanten der Paraproteinspezifität nachweisen. Lymphozyten mit Membran und intrazytoplasmatischem Paraprotein wurden als Intermediärformen der Plasmazellentwicklung gewertet. Möglichkeiten der Zellproliferationsstörung bei lymphoproliferativen Erkrankungen werden diskutiert.

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The Fibrinolytic Enzyme System in Malignant Lymphomas

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Abstract Components of the fibrinolytic enzyme system were measured in 40 patients with malignant lymphomas Fibrinolytic activity as assessed by the euglobulin clot lysis time, was reduced in the majority of patients The mean fibrinogen level was elevated in patients with Hodgkin s disease and reticulum cell sarcoma Four patients in the terminal phase of their illness had a marked reduction in plasminogen α_1 Antitrypsin and serum activation inhibitor levels were increased α_2 macroglobulin levels were normal No patient had conclusive evidence of disseminated intravascular coagulation

Key Words
Fibrinolysis
Hodgkin s disease
Lymphosarcoma
Reticulum cell sarcoma

There is abundant evidence that disseminated intravascular coagulation (DIC) may occur in malignant disease [7, 9, 18], and studies on alterations in coagulation and fibrinolysis in patients with cancer have been reported [17] Little information is available on changes in components of the fibrinolytic enzyme system in patients with malignant lymphomas This study examines the individual components of the fibrinolytic system in an unselected series of patients with malignant lymphomas with particular reference to the stage of the disease and the evidence for the presence of DIC.

Methods and Patients

Plasminogen activator was assayed by performing euglobulin clot lysis times by the method of Nilsson and Olow [11] the results were expressed by plotting the lysis times logarithmically against units of fibrinolytic activity 10 units being equated with a lysis time of 50 min

Plasminogen was measured by the caseinolytic technique of ALKJAERSIG *et al* [1] and the values expressed in Sherry units [2]

Fibrinogen was measured by a modification [12] of the method of RATNOFF and MENZIE [15]

Serum fibrin/fibrinogen degradation products (FDP) were assayed by the tanned red cell haemagglutination inhibition technique [8]

Activation inhibitor was assayed in serum using a standard clot system as described by BENNETT [3] One unit of inhibition is equivalent to that produced by 10^{-3} M ε-amino-caproic acid

α_1 Antitrypsin and α_2 macroglobulin levels were measured by single radial immunodiffusion [6] using Partigen plates obtained from Hoechst Behringwerke AG

Patients Forty patients with malignant lymphomas were studied. The diagnosis and type of malignant lymphomas were established by standard histological and haematological techniques, including biopsy of lymph node or liver and marrow aspiration Clinical staging into groups using the criteria of PETERS *et al* [14] was aided by lymphography in most patients and by laparotomy in a few No attempt was made to sub-divide the stages according to the presence or absence of general symptoms.

Twelve patients had Hodgkin s disease their age range was 19 to 57 years. Four had stage I or II disease 3 had stage III disease and 5 had stage IV disease Fourteen patients with lymphosarcoma ranged in age from 25 to 84 years. 2 had stage I or II disease 5 had stage III disease and 7 had stage IV disease. The remaining 14 patients had reticulum cell sarcoma, and were aged 19 to 81 years 2 had stage I or II disease 6 had stage III disease and 6 had stage IV disease

Six of the 40 patients, all with generalized disease, were receiving treatment at the time of study 2 patients were receiving prednisone a further 4 patients were on a course of antimitotic chemotherapy combined with prednisone The remaining patients were recently diagnosed and untreated at the time of venepuncture

Control values were obtained from healthy volunteers or patients convalescent from a variety of minor disorders Plasminogen activator levels were obtained from subjects who had been at rest in bed for at least 12 h

Results

The values for the components measured in control subjects are shown in table I In the case of blood components whose level is age-dependent (fibrinogen and α_2 macroglobulin) mean levels for different age-groups are shown

Plasminogen activator The patients with Hodgkin s disease and lymphosarcoma had a reduced mean level compared to control subjects (table II) The patients with Hodgkin s disease of stage IV had a lower mean level (2 0 units) than those of stage I and II (4 1 units) However considering patients with all types of reticulosis the stage of disease had little in

Table I Values for components of the fibrinolytic enzyme system in control subjects

	Number of subjects	Age years range	Age years mean	Concentration mean	Concentration SD
Plasminogen activator, units	40	18–79	49.0	4.6	1.4
Plasminogen casein units/ml	180	18–85	41.8	4.3	0.5
Fibrinogen, mg/100 ml	70	18–35	28.2	310	38
	75	36–55	45.3	347	51
	40	56–85	62.2	382	61
Fibrin degradation products, μg/ml	30	19–79	47.7	5.6	4.1
Serum activation inhibitor, units	22	19–85	54.6	3.4	1.0
α_1-Antitrypsin, mg/100 ml	80	21–85	49.3	275	48
α_2-Macroglobulin, mg/100 ml	30	19–59	32.3	290	50
	50	50–64	57.3	271	48
	20	61–85	66.0	262	43

Table II Values for components of the fibrinolytic enzyme system in patients with malignant lymphomas (mean ± SD)

	Hodgkin's disease n = 12	Lymphosarcoma n = 14	Reticulum cell sarcoma n = 14
Plasminogen activator, units	3.1 ± 1.3	3.2 ± 2.3	4.1 ± 2.7
Plasminogen, casein units/ml	3.9 ± 1.4	4.0 ± 0.9	3.9 ± 1.5
Fibrinogen, mg/100 ml	460 ± 176	378 ± 158	444 ± 153
Fibrin degradation products, μg/ml	7.3 ± 5.9	13.5 ± 11.9	8.9 ± 9.0
Activation inhibitor, units	4.5 ± 2.3	6.2 ± 3.1	4.8 ± 2.8
α_1-Antitrypsin, mg/100 ml	402 ± 84	352 ± 89	372 ± 133
α_2-Macroglobulin, mg/100 ml	245 ± 47	252 ± 57	243 ± 58

fluence on activator levels the mean level for the 18 patients with stage IV disease was 2.8 units compared to 3.2 units for the stage I and II patients.

Plasminogen The plasminogen levels, related to histological type and disease stage, are shown in figure 1. The majority of values lay within the normal range, but 8 patients, all with stage III or IV disease had reduced

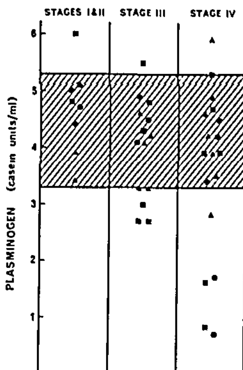


Fig 1 Plasminogen levels in patients with malignant lymphomas related to histological type and disease stage ● = Hodgkin's disease ▲ = lymphosarcoma ■ = reticulum cell sarcoma Shaded area represents mean ± 2 SD for control subjects

levels In 4 patients, the plasminogen concentration was markedly reduced, all these patients were in the terminal stage of their illness and are considered further below

Fibrinogen When age was taken into account, the patients with Hodgkin's disease had considerably elevated fibrinogen levels This was particularly marked in the 5 patients with stage IV disease (mean 500 mg/ 100 ml) The mean fibrinogen concentration of patients with lymphosarcoma was not significantly different from controls in the same age group, but there was a moderate rise in the levels in patients with reticulum cell sarcoma

α_1-Antitrypsin All histological types of malignant lymphoma had an elevated mean α_1-antitrypsin level This increase was present in all disease stages, but was most marked in patients with stage IV disease (mean

404 mg/100 ml) compared to a mean of 325 mg/100 ml for stage I and II patients

α_2-Macroglobulin In contrast to the elevated α_1-antitrypsin levels, the α_2 macroglobulin concentration did not differ from control levels in any type or stage of malignant lymphoma

Serum activation inhibitor The mean activation inhibitor levels were increased in all types of malignant lymphoma The increase was most marked in stage IV patients (mean 6 6 units) compared to 4 4 units for patients with stage I or II disease

Serum fibrin/fibrinogen degradation products The majority of FDP levels lay within the normal range A few patients had levels more than 2 standard deviations above the mean for controls (over 13 7 μg/ml) This was particularly seen in the stage IV patients, reflected in the higher mean level (14 5 μg ml) in these than in patients with less extensive disease (6 9 μg ml) Only 2 of the 40 patients, however, had a FDP level in excess of 20 μg ml

Reduced plasminogen levels Four patients had a marked reduction in the plasminogen concentration (under 2 0 casein units/ml) They were characterised clinically by being in the terminal stages of the disease The fibrinogen levels in these patients were within the normal range The platelet count was markedly reduced in 3 of the patients but the FDP level was not increased in these The remaining patient presented with purpura gangraenosa and was subsequently found to have reticulum cell sarcoma, in this patient the platelet count was normal at 176 000 μl, but the FDP level was elevated at 32 0 μg ml in association with reduced plasma fibrinolytic activity None of the 4 patients with a plasminogen level between 2 7 and 3 0 casein units ml had a FDP level in excess of 20 μg ml (mean 8 6 μg ml) a reduced platelet count or an increase in fibrinolytic activity

Haemorrhagic features Only 3 patients had haemorrhagic features at the time of study, all had a platelet count below 30 000 mm³ while none had an increase in the level of FDP

Discussion

Reported observations on the level of individual components of the fibrinolytic enzyme system in patients with malignant lymphoma are scanty Increased overall fibrinolytic activity in patients with Hodgkin's disease was described by ZARDI [19] while SCHULTZ and KNOBLOCH [16] reported

that fibrinolytic activity was normal in Hodgkin's disease, but increased in association with lymphosarcoma GIROLAMI and CLIFTON [5] using the euglobulin clot lysis time, found that fibrinolytic activity was increased in some 20% of patients with a reticulosis In the present study, we have found a tendency towards reduced plasminogen activator levels as assessed by the euglobulin clot lysis time In addition, we have observed an increased level of two of the inhibitors of the human fibrinolytic enzyme system, *α_1-antitrypsin with an antiplasmin action and an inhibitor of plasminogen* activation which is found during the coagulation of blood [4], both inhibitors showed a trend towards higher levels in more extensive disease

In a previous study we found 2 patients with Hodgkin's disease with a markedly reduced plasminogen level [10] In the present series of 40 unselected patients, 4 had a very low plasminogen level and a further 4 had a moderate reduction With a single exception all these patients had FDP levels within normal and we conclude that the low plasminogen was not the result of consumption secondary to disseminated intravascular coagulation The very low levels were associated with the terminal phases of the illness and we postulate that they result from impaired synthesis

Evidence for disseminated intravascular coagulation from a combination *of reduced plasminogen and fibrinogen levels and a low platelet* count with an increase in FDP was not conclusively present in any of the patients studied This is in contrast with the frequency of such changes in patients with acute leukaemia [13]

The clinical significance of the observed changes in components of the fibrinolytic system is uncertain The combination of low plasminogen activator level, increased levels of inhibitors, and raised fibrinogen concentration might be considered to predispose to thrombosis, but this complication was uncommon in the patients studied Haemorrhagic complica*tions were found only in those patients with thrombocytopenia*

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Die spontane, standardisierte Thrombozytenadhäsion und -aggregation in der Zählkammer

Eine einfache Orientierungsmöglichkeit über die Thrombozytenfunktionen aus kleinen Blutvolumina[1]

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Abstract An easy, economic and standardised method of orientation about the 3 most important platelet functions out of a small quantity of blood is described In order to examine the reliability of this method a comparative study between the aggregation results in the counting chamber and the platelet aggregation test (PAT) according to BREDDIN was carried out To obtain an exact determination of aggregation in the counting chamber not only the classification into aggregation grades (visual criterium) was taken but also after counting the 'free platelets the aggregation index (PAgI) according to REUTER *et al* was calculated The good concordance of the comparative results of these 2 technically totally different methods are discussed

Key Words
Adhesiveness and aggregation of platelet
Platelet aggregation inde
Platelet function
Thrombasthenia

Fur eine komplette Durchfuhrung von Thrombozytenfunktionsprufungen werden noch immer relativ grosse Blutvolumina benotigt Die zur Zeit brauchbaren Methoden sind daher mehr fur die Untersuchung grosserer Kinder und Erwachsener geeignet

Bei Neugeborenen sind Blutungen nicht selten bei etwa 3% der Kinder Am haufigsten sind intrakranielle und Darmblutungen, sowie Cephalhamatome [18] Bei einer allgemeinen Normo- bzw Hyperkoagulamie [1, 17, 26] sind in den ersten Lebenstagen die Einzelgerinnungsfaktoren herabgesetzt [17] In letzter Zeit kamen auch erste ausfuhrlichere

[1] Herrn Prof Dr J STRÖDER zum 60 Geburtstag gewidmet

Berichte über vorübergehende quantitative und qualitative Thrombozytenveränderungen bei Neugeborenen in den ersten Lebenstagen [15, 20, 21].

Besonders anfällig sind kranke Früh- und Neugeborene, deren Blutgefässe durch perinatale Hypoxie geschädigt sind. Bei der so entstandenen kapillären Fragilität ist, wie aus neueren Erkenntnissen hervorgeht [11, 27], die Rolle der funktionstüchtigen Thrombozyten unverkennbar. Die intrauterine oder perinatale Asphyxie führt zum perinatalen Schock. Blutdruckabfall bewirkt über Katecholaminausschüttung Vasokonstriktion der Arteriolen, Hypoxie, Acidose und zuletzt Gewebs- und Blutzellschädigung [18]. Es ist noch nicht bekannt, wie diese Vorgänge auf die Thrombozyten einwirken und ob sie vielleicht eine Freisetzungsreaktion (release reaction) mit visköser Metamorphose verursachen können. Bisher liegen nur Berichte vor, dass bei Früh- und Neugeborenen, die an einem Respiratory distress syndrome mit hyalinen Membranen *ad exitum* kamen, eine intravasale Gerinnung mit Verminderung der Plättchenzahl mehrfach nachgewiesen wurde [2, 16]. Daher kommt bei der Durchführung eines Gerinnungsstatus der gleichzeitigen Testung aller wichtigen Thrombozytenfunktionen immer grössere Bedeutung zu. Leider stehen dazu nur kleine Blutvolumina zur Verfügung und es gelingt auch nur sehr selten bei gleichzeitiger Schonung des kranken Kindes mehr als 3–5 ml Vollblut zu erhalten.

Da die Technik zur Messung der Thrombozytenadhäsivität in der silikonisierten Zählkammer zusätzlich ein Urteil über die Plättchenaggregation erlaubt [4], haben wir die Methode weiter ausgearbeitet. Es wird hier über Vergleichsuntersuchungen von Plättchenaggregationstest (PAT) nach BREDDIN [6–8] und spontaner Plättchenaggregation und -adhäsion in der Zählkammer [4, 24] berichtet, ferner über Plättchenausbreitung, die ebenfalls in der Zählkammer beurteilt werden kann.

Methodik und Untersuchungsgut

Untersucht wurde Zitratblut (Mischungsverhältnis 1:9), das den Probanden mit einer Kanüle Nr. 1 und einer Plastikeinmalspritze abgenommen wurde. Blut von Blutspendern liessen wir über eine Kanüle mit 1,2 mm Durchmesser und ein Stück Kunststoffschlauch in ein Plastikröhrchen mit Zitrat tropfen. Nach der Abnahme von Vollblut zur Plättchenzählung und zur Hämatokritbestimmung erfolgte sofortiges Zentrifugieren 10 min lang bei 800 U/min. Anschliessend Abpipettieren von plättchenreichem Plasma (PRP) und eventuelles Einstellen auf eine niedrigere

Plättchenzahl mit plättchenarmem Plasma (PAP), wenn die Plättchenzahl im PRP über 400 000 liegt oder die PRP-Menge nicht für die Untersuchungen ausreicht. Die Teste wurden normalerweise 45 min bis spätestens 1 h nach der Blutabnahme durchgeführt.

Thrombozytenzählung nach FEISSLY und LÜDIN [10], modifiziert nach DERLATH.

Adhäsionspräparate nach BREDDIN [4]. Wir achteten darauf, dass die verwendete Bürker-Kammer folgende Bedingungen erfüllt: sie muss nach dem Silikonieren bei Normalplasma eine gleichmässige Verteilung der adhäsiven Plättchen und Aggregate aufweisen. Sie darf nicht mehr als 6–8mal benutzt werden. Um eine gleichmässige Verteilung des PRP auf beide Netzteilungen zu erreichen, lassen wir – wie bei der Plättchenzählung – 2 Tropfen (auf jede Seite je einen Tropfen) unter das schon vorher festanliegende Deckglas fliessen. Die Kontaktzeit beträgt 10 min (Stoppuhr!), anschliessend 10maliges Eintauchen in 200 ml des Flüssigkeitsgemisches physiol. Kochsalzlösung + Na-Zitrat 3,13% im Mischungsverhältnis 10:1. Das Präparat wird nach Fixieren mit 37% Formol (5 min lang) einige Sekunden in Aqua dest. eingetaucht und dann luftgetrocknet. Aus der Mitte jeder Netzteilung wird je eine senkrechte und waagerechte Säule ausgezählt und das arithmetische Mittel gebildet (Kammermittelwert). Zunächst werden alle adhärenten Thrombozyten im Präparat gezählt, anschliessend die «freien Plättchen». Die Aggregate werden beschreibend als winzige (2–3 Plättchen), kleine (bis 5 Plättchen), mittlere (bis 12, höchstens 16 Plättchen) und grössere Aggregate angegeben. Die Häufigkeit und die Art der verschiedenen Aggregate im Präparat zusammen mit der Zahl der «freien Plättchen» werden bei der Beurteilung der Aggregationsstufe berücksichtigt. Ausserdem achtet man auf die qualitative Ausbreitung der Thrombozyten und das eventuelle Auftreten der viskösen Metamorphose, die an mehr oder weniger verwaschenen, substanzarmen und im phasenkontrastmikroskopischen Bild als Schatten wirkenden Formen zu erkennen ist. Beim Auftreten von kompakten Aggregaten (oder Agglutinaten) ist genaue Plättchenzählung nicht möglich. Dann werden die «freien Plättchen» gezählt und die kompakten Aggregate pro mm² bestimmt. Die Anregung zu unserem Vorgehen gab die Kammermethode von GROSS *et al.* [12]. Die lockeren Aggregate werden nicht gezählt.

Den Plättchenadhäsivitätsindex (PAI) errechnet man nach der Formel von BREDDIN [4].

Die Aggregationsgrösse wird folgendermassen bestimmt: von der Gesamtzahl der adhäsiven Plättchen wird die Zahl der «freien Plättchen» abgezogen. Der Plättchenaggregationsindex (PAgI nach REUTER *et al.* 24) kann anschliessend auf die gleiche Weise wie der PAI errechnet werden.

$$\text{PAgI} = \frac{(\text{Zahl der adhäsiven Plättchen pro mm}^2 - \text{Zahl der freien Plättchen pro mm}^2) \times 100}{\text{Zahl der Plättchen pro mm}^3 \text{ im Plasma}}$$

Zum Vergleich wurde neben dieser standardisierten Kammermethode parallel der *Plättchenaggregationstest* (PAT) durchgeführt nach der von BREDDIN [6–8] erarbeiteten Methode. Die Ergebnisse dieses Tests wurden nach visuellen Kriterien in Aggregationsstufen angegeben und mit den Aggregationsstufen in der Zählkammer verglichen. Als Anfangsstufe wurde die Stufe O genommen, weil einige Präparate überhaupt keine Aggregate aufwiesen. Ausserdem wurden Zwischenstufen

zum Beispiel 1,5 bei sonst schwer beurteilbaren Präparaten eingeführt. Die *Plättchenausbreitung* nach MARX *et al.* modifiziert nach BREDDIN und BÜRCK [3] wurde *ebenfalls routinemässig zum Vergleich durchgeführt.*

In dieser Vergleichsuntersuchung wurden die Befunde an 70 Personen ausgewertet. 19 davon waren gesunde Probanden (17 Erwachsene und 2 Neugeborene). Bei den 51 Patienten handelte es sich 4mal um eine verdächtige Blutungsneigung, 10mal um virale Erkrankungen (Influenza, Masern, Hepatitis), 12mal um eine Rh oder ABO-Inkompatibilität mit Hyperbilirubinämie, 6mal um unklare leichte Blutungen (Petechien, Hämatome, Nasenbluten, Erythrurie und Melaena). Je 2 mal bestand ein akutes Atemnotsyndrom des Neugeborenen, eine Thrombasthenie, ein M. v. Willebrand Jürgens und eine Thrombopenie (M. Werlhoff). Schliesslich waren vereinzelt Urämie, Heizölintoxikation, progressive Muskeldystrophie, Wiskott Aldrich Syndrom, septischer Abort mit Verbrauchskoagulopathie, rheumatisches Fieber, Colitis ulcerosa, Panarteriitis bzw. Zustand nach Nephrektomie, Glomerulonephritis, Gravidität und hereditäres Trophoderm (Milroy Syndrom) vertreten.

Ergebnisse

Bei dem *Vergleich der Plättchenaggregationsstufen* nach spontaner Adhäsion und Aggregation in der silikonisierten Bürker Kammer und

elle 1. Auswertung der Präparate, die einen Unterschied um mehr als eine Aggregationsstufe zeig

Kammer			PAI	PAgI	Aggregationsstufe		Diagnose (Jahre)	Bemerkung
Plättchenzahl × 1 000 mm³	adhäsive Plättchen mm³	freie Plättchen mm³			Kammer	PAT		
248	2 500	1 120	1	0,5	2	3,5	Unspez. Virusexanthem (6)	
332	8 000		1,46		2,5	5	Heizölintoxikation (2)	
233	2 550	1 240	1	0,5	2	4	Gravidität (31)	2,5 h nach Blutabnahme 4 C
476	9 960	1 790	2,3	1,9	3,5	5	Blutspender (58)	2 h nach Blutabnahme
336	120	170	0,03	0	0	2,5	Thrombasthenie (6)	• Sonderfall 1
325	0					0,5	Thrombasthenie (13)	• Sonderfall 2

nach Rotation des PRP im PAT zeigten 41 Personen (58,6%) eine vollständige Übereinstimmung. Einen Unterschied um ½ Aggregationsstufe wiesen 15 Präparate auf (21,4%). Bei dem klinisch und praktisch irrelevanten ½-Stufenunterschied können somit schon 80% der Präparate als übereinstimmend bezeichnet werden.

Bei 8 Fällen (11,4%) fand sich eine Differenz von einer ganzen Stufe. Um mehr als eine Stufe wichen 4 Ergebnisse ab (5,7%). Zwei Probanden (2,9%) waren «Sonderfälle», weil ihre Thrombozyten in der silikonisierten Zählkammer kaum oder nicht adhärierten (Tab. I), jedoch im PAT und in den Ausbreitungspräparaten an den silikonisierten Objektträgern fixiert blieben. Diese Thrombozyten waren zu fast 100% nicht ausgebreitet und zeigten mittels ihrer dünnen, nach Rotation des PRP länger gewordenen Fortsätze, wohl eine gewisse Aggregation. Es handelt sich dabei um zwei Fälle von Thrombasthenie (Abb. 5a, b).

Bei den Vergleichspräparaten, die einen Unterschied von ½ bzw. 1 Aggregationsstufe aufwiesen, hatten die PAT-Präparate 13mal und die Kammerpräparate 9mal die höhere Stufe.

Die Abbildungen 1a und b sowie 2a und b zeigen Übereinstimmung zweier Methoden. Die oben erwähnten 4 Ergebnisse, die in mehr als einer Stufe differierten, werden wegen dieser Besonderheit und unregelmässiger Testung in Tabelle I aufgeführt.

Die Neugeborenen mit akuter pulmonaler Insuffizienz zeigten bei den Vergleichsuntersuchungen nach visuellen Kriterien jeweils die Aggregationsstufe 1,5. Der PAgI betrug 0,48 bzw. 0,60.

Tabelle II. Plättchenzahl, -adhäsion und -aggregationsindex bei steigenden Aggregationsstufen in der silikonisierten Zählkammer (Mittelwerte und Standardabweichung)

Gruppe	Aggregationsstufe	Plättchenzahl × 1 000 mm³	Adhäsive Plättchen/mm²	PAgI	Zahl der Fälle
1	0–0,5	219 ± 104	2 210 ± 1 093	0,11 ± 0,01	12
2	1–1,5	282 ± 94	3 055 ± 1 370	0,61 ± 0,33	16
3	2–2,5	336 ± 144	6 033 ± 2 687	1,31 ± 0,59	12
4	3–4	304 ± 123	8 181 ± 3 178	2,28 ± 0,88	15
				Insgesamt	55[1]

[1] Bei den restlichen 15 Fällen wurden die «freien Plättchen» nicht gezählt, folglich konnte PAgI nicht bestimmt werden.

Abb. 1 6 Jahre alter Junge mit progressiver Muskeldystrophie. Aggregationsstufe 3

Abb. 2 Erwachsener Blutspender unter 45 Jahre. Aggregationsstufe 1,5. *a* Adhäsion und Aggregation in der silikonisierten Zählkammer × 210. *b* Plättchenaggregationstest × 320

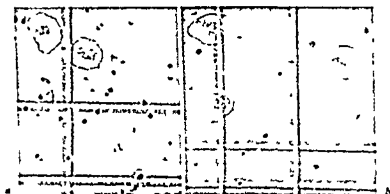


Abb. 3 10jähriger Knabe mit Hepatitis epidemica. Ausgebreitete Riesenformen in der silikonisierten Zählkammer. Adhäsionstest nach Breddin × 320



Abb 4 Kaninchenthrombozyten
Abb 5 Thrombozyten eines 6jährigen Mädchens mit Thrombasthenie *a* Plättchenausbreitung *b* Plättchenaggregationstest ×640

Die Ergebnisse der *Adhäsion und Aggregation in der Zählkammer* wurden nach Aggregationsstufen in 4 Gruppen eingeteilt wie aus der Tabelle II zu ersehen ist Im Vergleich mit den Aggregationsstufen (0–4) zeigt der PAgI ein fast lineares Verhalten, bei gleichzeitiger Zunahme auch der adhäsiven Thrombozyten Sowohl die pathologischen Fälle als auch die Befunde der Normalpersonen verteilen sich fast regelmässig auf die ersten 3 Gruppen Die 4 Gruppe mit pathologischer Aggregation und relativ hoher Adhäsion enthält nur 2 regelrecht durchgeführte Teste (45 min bis 1 h nach der Blutabnahme Zimmertemperatur) die jedoch von klinischer Bedeutung sind (eine progressive Muskeldystrophie und eine Ovulationshemmermedikation) Die übrigen Ergebnisse in dieser Gruppe stammen aus früheren Untersuchungen bei Normalpersonen unter variablen Zeit- und Temperaturfaktoren Sie ergaben wegen der späten Durchführung (erst 2 h nach der Blutabnahme zum Teil wurde das PRP bei 4 °C gelagert) pathologische Werte

Die *Plättchenausbreitung* kann man in der Zählkammer dank der Einstellung auf niedrigere Plättchenzahlen und sorgsamen Silikonierens der Kammer meistens gut beurteilen. Die Kontaktzeit von 10 min gewährleistet eine ausreichende Differenzierung aller Ausbreitungsformen. Bei Untersuchung von 5 Patienten mit Hepatitis epidemica (4 Jugendliche, 9–14 Jahre und eine Frau, 23 Jahre alt) wurden mit dieser Methode für diese Erkrankung vielleicht charakteristische Ausbreitungsformen der Zellen, die grösser als normale Riesenthrombozyten sind, beobachtet. So häufig sind diese Formen in der Kammer, bei relativ kurzer Kontaktzeit, bis jetzt bei keinem anderen pathologischen Zustand gesehen worden (Abb. 2).

Im *Tierversuch* adhärieren Kaninchenthrombozyten in der Zählkammer mit wechselnder Intensität von Tier zu Tier (PAI von 0,12 bis 2,8). In den gefärbten (Ausbreitung, PAT) Präparaten haben sie wenige, dünne Fortsätze, sind meistens rund und werden auf den silikonierten Objektträgern gut fixiert. Es kommt dabei zu Aggregation, meistens mittels der Fortsätze. Da Kaninchenplättchen keine Ausbreitung zeigen, kann man sie in PAT- und Ausbreitungspräparaten von menschlichen Plättchen bei Thrombasthenie Kranken morphologisch kaum unterscheiden (Abb. 4a, b).

Diskussion

Bei dem Versuch, an kranken Früh- und Neugeborenen Thrombozytenteste durchzuführen, taucht immer wieder das Problem einer ausreichenden und schonenden Blutabnahme bei diesen Kindern auf. Oft erhält man bei ihnen nur eine geringe Blutmenge (ca. 3 ml), die etwa 0,7 ml PRP ergibt.

Aus der Vergleichsuntersuchung (80% übereinstimmende Befunde) zwischen den Aggregationsstufen im PAT und nach der Kammermethode sowie durch statistische Auswertung der mit der Kammermethode erhobenen Befunde, glauben wir, auch nach Vergleich mit den Ausbreitungspräparaten, eine standardisierte, genaue und schnelle Orientierungsmöglichkeit über die 3 wichtigsten Thrombozytenfunktionen gefunden zu haben. Insbesondere für den Pädiater, ist die Methode wichtig, weil die wesentliche Vereinfachung darin besteht, dass zur Beschickung der Bürker-Kammer nur 2 Tropfen PRP gebraucht werden statt 1,5 ml [5–8] oder 0,6 ml (Wrisso et al., unveröffentlichte experiment. Ergebnisse) allein für den Aggregationstest. So bleibt PRP für weitere wichtige Thrombozytenprü-

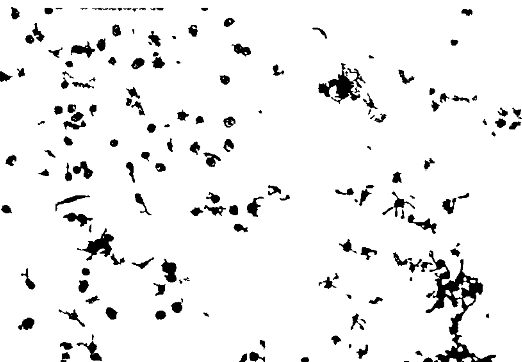


Abb 4 Kaninchenthrombozyten

Abb 5 Thrombozyten eines 6jährigen Mädchens mit Thrombasthenie *a* Plättchenausbreitung, *b* Plättchenaggregationstest ×640

Die Ergebnisse der *Adhäsion und Aggregation in der Zählkammer* wurden nach Aggregationsstufen in 4 Gruppen eingeteilt, wie aus der Tabelle II zu ersehen ist Im Vergleich mit den Aggregationsstufen (0–4) zeigt der PAgI ein fast lineares Verhalten, bei gleichzeitiger Zunahme auch der adhäsiven Thrombozyten Sowohl die pathologischen Fälle als auch die Befunde der Normalpersonen verteilen sich fast regelmässig auf die ersten 3 Gruppen Die 4 Gruppe mit pathologischer Aggregation und relativ hoher Adhäsion enthält nur 2 regelrecht durchgeführte Teste (45 min bis 1 h nach der Blutabnahme, Zimmertemperatur), die jedoch von klinischer Bedeutung sind (eine progressive Muskeldystrophie und eine Ovulationshemmermedikation) Die übrigen Ergebnisse in dieser Gruppe stammen aus früheren Untersuchungen bei Normalpersonen unter variablen Zeit- und Temperaturfaktoren Sie ergaben wegen der späten Durchführung (erst 2 h nach der Blutabnahme, zum Teil wurde das PRP bei 4 °C gelagert) pathologische Werte

Die *Plättchenausbreitung* kann man in der Zählkammer dank der Einstellung auf niedrigere Plättchenzahlen und sorgsamen Silikonierens der Kammer meistens gut beurteilen. Die Kontaktzeit von 10 min gewährleistet eine ausreichende Differenzierung aller Ausbreitungsformen. Bei Untersuchung von 5 Patienten mit Hepatitis epidemica (4 Jugendliche, 9–14 Jahre und eine Frau, 23 Jahre alt) wurden mit dieser Methode für diese Erkrankung vielleicht charakteristische Ausbreitungsformen der Zellen, die grösser als normale Riesenthrombozyten sind, beobachtet. So häufig sind diese Formen in der Kammer, bei relativ kurzer Kontaktzeit, bis jetzt bei keinem anderen pathologischen Zustand gesehen worden (Abb. 2).

Im *Tierversuch* adhärieren Kaninchenthrombozyten in der Zählkammer mit wechselnder Intensität von Tier zu Tier (PAI von 0,12 bis 2,8). In den gefärbten (Ausbreitung, PAT) Präparaten haben sie wenige, dünne Fortsätze, sind meistens rund und werden auf den silikonierten Objektträgern gut fixiert. Es kommt dabei zu Aggregation, meistens mittels der Fortsätze. Da Kaninchenplättchen keine Ausbreitung zeigen, kann man sie in PAT- und Ausbreitungspräparaten von menschlichen Plättchen bei Thrombasthenie-Kranken morphologisch kaum unterscheiden (Abb. 4a, b).

Diskussion

Bei dem Versuch, an kranken Früh- und Neugeborenen Thrombozytenteste durchzuführen, taucht immer wieder das Problem einer ausreichenden und schonenden Blutabnahme bei diesen Kindern auf. Oft erhält man bei ihnen nur eine geringe Blutmenge (ca. 3 ml), die etwa 0,7 ml PRP ergibt.

Aus der Vergleichsuntersuchung (80% übereinstimmende Befunde) zwischen den Aggregationsstufen im PAT und nach der Kammermethode sowie durch statistische Auswertung der mit der Kammermethode erhobenen Befunde, glauben wir, auch nach Vergleich mit den Ausbreitungspräparaten, eine standardisierte, genaue und schnelle Orientierungsmöglichkeit über die 3 wichtigsten Thrombozytenfunktionen gefunden zu haben. Insbesondere für den Pädiater, ist die Methode wichtig, weil die wesentliche Vereinfachung darin besteht, dass zur Beschickung der Bürker-Kammer nur 2 Tropfen PRP gebraucht werden statt 1,5 ml [5–8] oder 0,6 ml [WITTIG *et al.*, unveröffentlichte experiment. Ergebnisse] allein für den Aggregationstest. So bleibt PRP für weitere wichtige Thrombozytenprü-

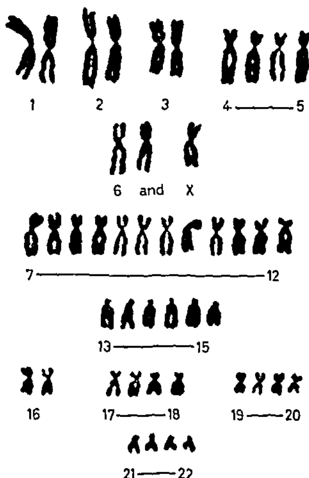


Fig 1 This karyotype shows a 45 XO pattern consistent with Turner's syndrome 18 of 50 cells counted showed this pattern

At 1 year of age, the patient was hospitalized with anemia and a spontaneous hemothorax (the only major bleeding episode of her life) She required blood transfusions and infusions of factor IX concentrates

At 2 years of age, buccal smears revealed only 3/200 cells to be chromatin positive Karyotypes were performed, of 50 cells analyzed, 32 were XX (ring) (fig 1), and 18 were XO (fig 2) These findings are indicative of a mosaic Turner's syndrome Mother and father had normal karyotypes

At a recent examination, the patient appeared as an alert, active, normal 3-year-old girl The height and weight were both in the 3rd percentile Multiple ecchymoses were present Epicanthal folds were noted and the nipples appeared somewhat widely spaced There was no webbed neck, cubitus valgus, low-set ears, low posterior hairline, shield chest or heart murmur The remainder of the physical examination was normal Hemoglobin 13 2 g%, hematocrit 38%, and factor IX less than 1%

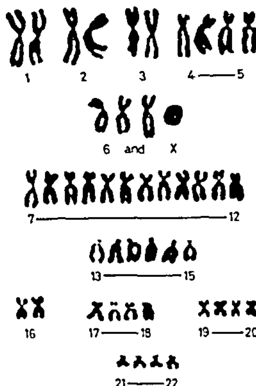


Fig 2 This karyotype shows a normal number of 46 chromosomes. The second X chromosome is a ring form. 32 of 50 cells counted showed this pattern

Discussion

Mosaic Turner's syndrome is a rare disorder in which the karyotype is usually XX/XO Physical features of this disorder are variable In 2 reported cases, the patients were phenotypically normal [2, 13] Other cases have demonstrated few of the characteristics of classic Turner's syndrome [11-13] One case of mosaic Turner's syndrome with classic hemophilia (factor VIII deficiency) has been reported [2] The present case is different from other reported cases as one X chromosome in the 46-chromosome karyotype was a ring form

Females can manifest X linked recessive disorders by one of several mechanisms Rare examples of homozygous females with X linked re-

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Abnormal Factor X (Factor X Friuli) Coagulation Disorder

First Report of a Case Outside Friuli

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Abstract A new case with the abnormal factor X (factor X Friuli) coagulation disorder is presented a 32 year old female who was born outside Friuli and who complained of a bleeding tendency since childhood The main laboratory features are prolonged prothrombin time prolonged partial thromboplastin time abnormal thromboplastin generation and normal Stypven cephalin clotting time The prothrombin time and the thromboplastin generation were corrected by the addition of normal serum Factor X was low only when assayed using whole tissue or partial thromboplastin It was normal using the Stypven cephalin mixture The cross-over electrophoresis showed a normal factor X band in the patient s plasma, whereas no band was present in Mr Stuart s plasma The father and a cousin of the patient are heterozygotes for the abnormality This is the first family reported to have the abnormal factor X (factor X Friuli) disorder outside Friuli

Key Words
Abnormal factor X
Bleeding disorders
Blood coagulation

Classical factor X deficiency was first recognized in 1956 and 1957 [1 14 15 17] A congenital hemorrhagic condition due to the presence of an abnormal factor X was described by us in 1969 and 1970 [6–8] This abnormal factor X cannot or can be activated only very slowly by whole tissue or partial thromboplastins whereas it can still be normally activated by Russell s viper venom (RVV) The eponym Friuli was attached to this abnormal factor X because all patients so far described were born in the northeastern Italian region called Friuli All 9 patients came from an isolated valley in the western Friuli area [11 12] Another patient with a factor X defect and a normal RVV cephalin clotting

time was seen recently in France but the diagnosis has not yet been fully established [3].

This is the first report of a patient found to have the same coagulation disorder but with a different geographical and familiar extraction.

Material and Methods

Material and methods have been described in detail elsewhere [7, 8]. Only new data will be given herein.

Lyophilized factor X deficient plasma was supplied by Dade Laboratories. Factor II in the one-stage system was assayed using Stago factor II reagent to which normal serum was added in a 2:10 proportion. Anti factor X antiserum was kindly supplied by Dr. D. Aronson of the National Institutes of Health, Bethesda, Md. Cross-over electrophoresis was carried out according to a modification of the method proposed by Bussard [illegible].

Behringwerke agar was dissolved in Michaelis buffer at pH 8.3 at the concentration of 1.5%. The agar solution was then stratified on 2.5 × 7.5 cm slides, the thickness of the agar was 2 mm. Wells of 7 mm diameter were prepared, the distance between the inner edges of two wells was 2.2 cm. The amount of the test plasma introduced in the well of the cathodic side was 0.065 ml. The amount of

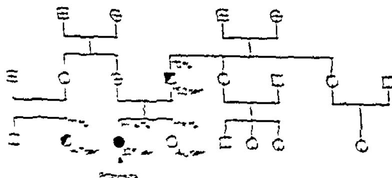


Fig. 1. Family pedigree. The upper number on the right side of each square or circle represents the prothrombin time. The lower number refers to the factor X level. ● = homozygote symptomatic; ◧ ◐ = heterozygote asymptomatic or mildly symptomatic; ○ = studied, normal; ⊜ ☰ = tested, presumably normal; □ ○ = not studied, presumably normal.

Table I Coagulation studies

Test	Patient	Normal values
Bleeding time, min	5	2–5
Clot retraction	complete after 4 h	complete after 12 h
Glass clotting time, min	13	5–10
Recalcification time, sec	465	100–180
Cross recalcification time (+0 05 ml of normal plasma), sec	137	–
Thromboplastin generation test, sec	32 in 6 min	< 16 in 6 min
Prothrombin consumption, %	50%	90
Partial thromboplastin time, sec	72 5	45
Prothrombin time, sec	33 5	13
Stypven-cephalin clotting time, sec	17	15
Factor II+X using a S/C mixture, sec	28	30
Factor II (one stage) %	100	85–120
Factor II (two stage) %	100	85–120
Factor V, %	90	85–120
Factor VII, %	135	85–120
Factor VIII, %	120	60 150
Factor IX, %	100	70–150
Factor X	see table III	–
Factor XI, %	80	60–130
Factor XII, %	100	60 130
Fibrinogen, mg%	400	250–450
Euglobulin lysis time, h	20	10–30
Thrombin time, sec	19	18–25
Th r, mm	34	10–20
K, mm		6–12
ma, mm		50–66

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Table II Prothrombin time (PT) correction studies

Mixture, equal parts	PT, sec	Reference plasma PT, sec	Comment
Proposita plasma	33.5	–	
Proposita plasma + normal serum	18.0	–	
Proposita plasma + adsorbed normal plasma	40.0	–	
Proposita plasma + factor II deficient plasma	16.0	20.0	frozen plasma, personal case
Proposita plasma + factor VII	19.0	53.7	lyophilized plasma received from Dr Owen
Proposita's plasma + factor X deficient plasma	38.9	72.5	dade lyophilized factor X deficient plasma
Proposita's plasma + plasma of another patient with the abnormal factor X disorder	33.5	33.6	frozen plasma, personal case

found between this patient and any of the other patients known to have the factor X Friuli disorder.

The patient has noticed easy bruising since childhood. Menstruations were normal most of the time, but occasionally have been abundant. Epistaxis has never occurred. At the age of 12 and again at 16 the patient presented bleeding after tooth extractions. She was not transfused but on the first occasion had to be admitted to a local hospital where local therapy was applied. At the age of 19 the patient presented a posttraumatic fracture of the left tibia. A cast was applied and the fracture healed in normal time with no sequel.

At the age of 21 the patient underwent surgery for acute appendicitis. She was not known then to be a bleeder and no preoperative transfusion therapy was given. In the immediate postoperative period bleeding was massive and a huge abdominal muscle hematoma formed. Routine coagulative tests were carried out and a tentative diagnosis of prothrombin complex factors deficiency was made. The patient was transfused with several units of fresh whole blood or plasma and the hematoma slowly subsided.

At the age of 22 the patient presented excessive bleeding after another tooth extraction. Bleeding subsided after 10 h but no transfusion therapy was given. At the age of 25 the patient had to be hospitalized because of bleeding after two tooth extractions. On this occasion bleeding stopped only after the transfusion of three units of whole blood.

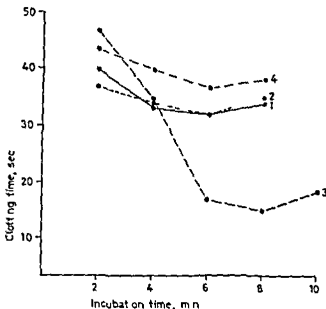


Fig 2 Thromboplastin generation test Curve 1 (basal curve) was obtained from a generation mixture containing 0 2 ml of patients' serum and 0 2 ml of adsorbed patient plasma Curve 2 refers to a generation mixture containing 0 2 ml of patients' serum and 0 2 ml of adsorbed normal plasma Curve 3 was obtained from a mixture containing 0 2 ml of normal serum and 0 2 ml of patient s adsorbed plasma The other components of the generation mixture in these 3 cases were 0 2 ml of a nonactivated cephalin preparation (Platelin) and 0 2 ml of a 0 025 M $CaCl_2$ solution Curve 4 refers to an incubation mixture containing 0 2 ml of patient s serum, 0 2 ml of patient s adsorbed plasma 0 2 ml of a Stypven-cephalin preparation (Diagen) and 0 2 ml of the usual $CaCl_2$ solution

Coagulation studies The results are summarized in table I The prothrombin time was prolonged and was corrected by normal serum and the plasma of patients with hypoprothrombinemia parahemophilia and factor VII deficiency Adsorbed normal plasma factor X deficiency and Friuli plasma failed to correct the abnormality (table II) The prothrombin proconvertin test partial thromboplastin time, prothrombin consumption and thromboplastin generation test were abnormal

The substitution of the patient s serum with normal serum in the thromboplastin generation test system corrected the abnormality (fig 2) The inclusion of Stypven-cephalin in the generation mixture failed to correct the defect (fig 2) The Stypven cephalin clotting time was normal or near normal The factor II+factor X complex using a Stypven cephalin mixture was also normal The factor X level was low only when a whole tissue or partial thromboplastin was used in the assay In contrast, it was normal or near normal when a S C mixture was used

All other clotting factors were found to be within normal limits Vascular and platelet tests were normal and there was no hyperfibrinolysis The thromboelastogram showed a prolonged r and k and a normal ma (fig 3)

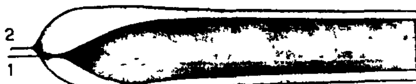


Fig 3 Thromboelastographic pattern The first tracing is the basal tracing The second tracing was obtained after the addition of 0 1 ml of a 10^{-4} ellagic acid solution to 0 9 ml of patient's platelet rich plasma The addition of the compound has partially corrected the abnormality

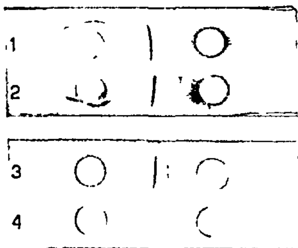


Fig 4 Cross-over electrophoresis of propositus plasma (1), normal plasma (2), plasma of another patient with this disorder (3), and Mr Stuart s plasma (4). A major or factor X band is evident for 1, 2 and 3 In 4 no such band is visible Smaller and lighter bands are visible in all plasmas. These are due to secondary activities of the antiserum used and may be disregarded

The cross-over electrophoresis showed that factor X Friuli has the same mobility as normal factor X. No factor X band was seen in Mr Stuart plasma (Fig 4)

Discussion

The main criteria for the diagnosis of the factor X Friuli disorder are prolonged prothrombin time, prolonged partial thromboplastin time cor-

Table III Factor X assay in % as determined with different substrates and thromboplastins

Substrate	Tissue thromboplastin	Cephalin	Stypven-cephalin mixture
Charcoal filtered ox plasma	14	11	60
Factor X deficient plasma + adsorbed normal plasma	8	10	70

rected by the addition of serum and normal Stypven cephalin clotting time Our patient fully meets these criteria

The lack of correction of the proposita's prothrombin time upon mixing her plasma with the plasma of another patient with this disorder, the factor X assays and the immunological studies confirmed the diagnosis The partial correction of the thromboelastographic tracing by the addition of ellagic acid is not surprising since it was already seen for the plasma of other patients with the same defect It is a nonspecific effect due to the first stage activating capabilities of this compound [8] The mobility of this abnormal factor X in a cross over electrophoresis system is identical to that of normal factor X This observation confirms previous data on the subject concerning the plasma of other patients with this disorder [13]

The hereditary pattern found in this patient is similar to that observed in the other patients with the same coagulation abnormality [8, 9] The father of our proposita is a heterozygote for the defect, even though he has always been asymptomatic The patient s cousin is a heterozygote too and has also had excessive bleeding after tooth extractions Occasional undue bleeding after surgical procedures has been noted in heterozygotes of other families with this disorder [8, 9] The condition is transmitted as an autosomal incompletely recessive trait in a way similar to what is described for classical factor X deficiency (9, 14, 16)

This is the tenth patient with this peculiar coagulation disorder so far described Nine patients were studied by us during 1968 and 1969 However, the present patient has no relationship with the other patients A total number of 10 or 11 patients, if the case from France will be confirmed does certainly not represent a large number However, it acquires a sure meaning when one recalls that since the year of 1956 only

about 20 cases of classical factor X deficiency have been described in the world literature [10]

It is likely that the mutation responsible for this factor X abnormality is more widespread than originally thought As already suspected by us it is likely that many cases of factor VII deficiency described in the past were indeed cases of abnormal factor X disorder

The normality of the Stypven or S C clotting time in the presence of a prolonged prothrombin time is the most commonly quoted criteria for a factor VII deficiency It is clear now that this assumption is not correct A normal Stypen-cephalin clotting time does not rule out a factor X defect due to an abnormal molecule The importance of factor X abnormalities in coagulation studies has been emphasized recently also by other authors [4 5]

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Acta Haemat 49 123–127 (1973)

Hypofibrinogenemia after *Echis colorata* Bite in Man

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Abstract An 18 year-old man was bitten by *Echis colorata* in his left ankle The patient was followed for 4 days without any therapy and coagulation studies proved fibrinolysis without disseminated intravascular coagulation

Key Words
Blood coagulation
Echis colorata bite
Fibrinolysis
Hypofibrinogenemia
Snake venom

Hypofibrinogenemia after *Echis colorata* bite is common [1 2] and the thrombocytopenia with the effect of the venom's hemorrhagin causes bleeding [3] The mechanism of disseminated intravascular coagulation (DIC) with secondary fibrinolysis [2 3] might explain the clinical and laboratory findings We followed a patient very closely and did not find DIC although the hypofibrinogenemia was very remarkable

Case History

An 18 year-old male was seen 15 h after he was bitten by *Echis colorata* in his left ankle The pertinent physical finding was edema of the ankle without oozing from the bitten area

On admission, hemoglobin 13.6 g%, leucocytes 8,000 with a normal differential platelets 136,000 and ret 0.2% Urinanalysis was normal and blood sugar urea, albumin, globulin cholesterol SGOT SGPT bilirubin and alkaline phosphatase were normal as well

The patient did not receive any therapy for the first 4 days in the hospital On the 5th day after being admitted to the hospital he received antivenin therapy (Rogoff Institute) and had anaphylactic shock He recovered after he received ultracorten (50 mg) and had no other complications

Fibrinogen which is needed in the 'plasmatic atmosphere' of the platelets for aggregation to occur [13] was non-detectable in the patients plasma This fact does not rule out the possibility that the platelets did have fibrinogen on their membrane, but we could not prove this point

Platelet aggregation was normal when FSP were found to be high in the serum as well as when FSP came down to normal levels It has already been described that FSP inhibit platelet aggregation [14], those were the low molecular weight fractions of FSP, but other fractions were found to enhance aggregation [15] Moreover fibrin monomers can form with FSP soluble complexes that induces platelet aggregation [16]

We had the unique opportunity to follow the natural course of *Echis colorata* bite in man without any therapy for the first 4 days and we can confirm the fact that no DIC was found

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H. MARTIN und L. NOWICKI (Hrsg.): Synthese, Struktur und Funktion des Hämoglobins. Lehmanns, München 1972. 402 pp., 194 fig., 64 tab.; DM 98.–.

Der vorliegende Band gibt die Ergebnisse des internationalen Symposiums vom 22. bis 24. April 1972 wieder, zu dem sich führende Biochemiker, Molekularbiologen und Hämatologen in Bad Nauheim zusammengefunden haben. Er vermittelt eine ausgezeichnete Übersicht über den derzeitigen Stand der Hämoglobinforschung. Einleitend enthält er Beiträge über die Kinetik der Erythropoese (DUPLAN, BOLL) und deren Regulation (KUBANEK) sowie über die Hämoglobinbildung, ihre molekularen Voraussetzungen, ihre zytochemischen Gesichtspunkte und ihre Steuerung (DE LA CHAPELLE, LÖFFLER, MÜLLER, NECHELES, NEUWIRT und POŇKA, ARNSTEIN, HUNT) unter besonderer Berücksichtigung der Proteinsynthese und der Rolle von Ribosomen, DNA und RNA (HUNT, HUNTER und JACKSON, ANDERSON, HUNTER et al., HARDESTY et al., BERMEK). Als einer der Hauptteile folgt die Darstellung der neuesten Erkenntnisse über die Hämoglobinstruktur mit den faszinierenden Konsequenzen, die dieses Modell für die gesamte Biologie und Pathophysiologie darbietet (HUBER, WINTERHALTER, GERSONDE, RUDLOFF, FITCH, HUISMAN und SCHROEDER, STEINHEIDER et al., WEATHERALL und CLEGG, RACHMILEWITZ und HARARI, KONIGSBERG et al.). Die nächsten Beiträge sind der Physiologie des roten Blutfarbstoffes und den Bedingungen des O_2-Transportes gewidmet (BARTELS und BAUER, GARBY, MAY und HUEHNS, BONHARD). Wie in den vorhergehenden Vorträgen weiss der Leser die Ausrichtung auf die Grundlagenforschung, die instruktiven Darstellungen und die Hinweise auf die Klinik zu schätzen. Dieses Kapitel schliesst den ersten, etwa zwei Drittel des Symposiums umfassenden, vorwiegend pathophysiologischen Teil und leitet über zum letzten Drittel, dessen Referate sich in erster Linie an den Kliniker wenden (NETH, KLEIHAUER, JONXIS, KOHNE, RIEGEL und VERSMOLD, BETKE, LEHMANN, NOWICKI, STICH). Es handelt sich um Übersichten, die auch für den Nicht-Hämatologen viel Interessantes bieten und gleichsam die praktischen Konsequenzen der im ersten Teil enthaltenen Ergebnisse darbieten.

Die Lektüre dieses Kongressberichtes ist ein Genuss und vermittelt sowohl dem theoretisch interessierten Biologen als auch dem Kliniker neuestes Wissen und zahlreiche Anregungen. Wenn BETKE in seinem Referat über Methämoglobinämien das Hämoglobin als ein «strukturell und funktionell wahrhaft bewundernswertes Gebilde» bezeichnet, so kann dies als Motto für das gesamte Symposium dienen. Hervorzuheben ist die Kurzfristigkeit der Publikation, wie sie für die Information über ein in rascher Entwicklung begriffenes Gebiet wie die Hämoglobinforschung beinahe Voraussetzung ist. Damit ist es wohl zu erklären, dass leider auf ein Stichwortverzeichnis verzichtet wurde – der einzige Nachteil dieser sonst ausgezeichneten Publikation.

H. LÜDIN, *Basel*

Acta Haemat. 49: 129–141 (1973)

Interrelations between Diagnostic Criteria of the Iron Status and 50-mg Ferrous Iron Absorption in Iron-Replete and Iron-Deficient Subjects[1]

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Abstract. In comparison to other criteria of iron status, higher degrees of association have been computed between the diffuse Prussian blue reaction of the bone marrow macrophages and intestinal ferrous iron absorption, when test doses of 0.56 mg, 50 mg in solution, and 50 mg in quick release capsules were given to fasting iron replete controls and iron-deficient subjects in ^{59}Fe absorption whole body retention tests. The variability of the results increased with rise of the test doses. Antecedent oral iron substitution and unallowed intake of food depressed ferrous iron absorption considerably.

Key Words
Bone marrow iron
Iron absorption
Iron deficiency
Iron status

A growing number of methods is used to define the normal iron status, to recognize the different stages of iron deficiency, and to assess the potentials of iron supplementation more precisely. In a large body of data covering a wide range of normality and disease, the degrees of association between the diagnostic variables have been determined by regression, correlation, and factor analysis [8]. A close negative correlation has been computed between the diffuse Prussian blue reaction of macrophages in bone marrow squash preparations [10] and intestinal iron absorption, when 0.558 mg of Fe^{++} + 17.6 mg of ascorbic acid were given to strictly fasting subjects 10 h before and 2 h after the application of the test dose in ^{59}Fe absorption whole body retention tests [13, 15]. The purpose of this paper is to present results of correlation analyses which include the administration of 50-mg test doses [14, 15] in solutions of 0.01 N HCl and in quick release capsules as used in oral iron supplementation. The

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findings relevant to oral iron therapy are compared in iron replete individuals and subjects with prelatent, latent, and manifest iron deficiency [9] Smaller series concern the inhibiting effect of unallowed intake of food and antecedent oral iron therapy on ferrous iron absorption Two subsequent papers will deal with the efficacy of quick release iron capsules given apart from meals as far as possible in patients with posthaemorrhagic and chronic haemorrhagic iron deficiency [11, 12]

Subjects and Methods

Correlation analyses (table I) Intestinal absorption of 50 mg of Fe^{++} in solutions of 0 01 N HCl has been examined in 103 subjects (table I) 27 normal iron replete controls 26 subjects with *prelatent (storage) iron deficiency* 10 with latent (storage + plasma) iron deficiency 13 with manifest (anaemic) iron deficiency 5 iron replete subjects after partial gastrectomy 15 with Hodgkin s disease and 7 with chronic infections Tests with 50 mg of Fe^{++} in quick release iron capsules were performed in 146 individuals (table I) 16 normal iron replete controls 48 subjects with prelatent iron deficiency 36 with latent iron deficiency 40 with manifest iron deficiency 6 with Hodgkin s disease with normal iron stores. For studies presented in table II and figure 2 somewhat higher numbers of cases could be evaluated The iron deficiency was of varied origin [9] Further details concerning table III and figure 3 are given under Results

The following variables of the *iron status* (filling state of the main easily accessible iron compartments of the body) have been studied with methods described in detail previously [8–10 13–15] Venous blood was taken in the morning after fasting overnight haemoglobin (cyanhaemiglobin) microhaematocrit red blood cells (Coulter counter model B and F) calculations of mean cellular haemoglobin (MCH) and mean cellular volume (MCV) Serum iron (bathophenanthrolin) total iron binding capacity (TIBC magnesium carbonate) calculation of transferrin saturation and unsaturated iron binding capacity (UIBC)

Prussian blue staining of bone marrow smears These contain thick squash preparations of larger fragments [10] counting of sideroblasts separate cytochemical grading (0 to 6 +) of 3 morphologically distinct types of nonhaeme iron in macrophages (diffuse iron polymorphous haemosiderin particles and uniform small sized Prussian blue granules) In every case slides fixed in the vapour of neutralized formaldehyde 35% (5 min) and stained with 100 ml of potassium ferrocyanide 1%+1 ml of hydrochloric acid 25% (15 min at room temperature) have been compared with slides fixed in methylalcohol (5 min) stained with 100 ml of potassium ferrocyanide 1%+1 ml of hydrochloric acid 25% (15 min at room temperature) and counterstained with aqueous nuclear fast red 0 01% (5 min)

Intestinal iron absorption This has been determined by HEINRICH and his coworkers [13–15] in a collaborative program [8–12] by means of ^{59}Fe absorption whole-body retention tests using a 4 π-large volume radioactivity detector with liquid scintillator [13] The subjects to be examined were asked several times to remain

fasting over night and 2 h after application of the test doses in order to avoid alimentary inhibition of ferrous iron absorption [8] Every individual received at first 0.558 mg of $^{59}Fe^{++}$ (10 μmol = 0.05–0.20 μCi) + 17.6 mg (100 μmol) L (+) ascorbic acid dissolved in 0.01 N HCl Further tests were performed at appropriate intervals [15] Solutions of 50 mg of $^{59}Fe^{++}$ + 150 mg of L (+) ascorbic acid have been applied in 0.01 N HCl [14] All liquid test doses had been frozen at −20 °C after preparation and thawed immediately before use The quick release iron capsules (Eryfer®, Hoechst, Frankfurt) contained 50 mg of ferrous iron (152 mg of ferrous sulfate H_2O labeled with ^{59}Fe) + 222 mg of L (+) ascorbic acid + 84 mg of Na HCO_3

During therapy, one quick release iron capsule was given in the morning 30–60 min before breakfast and a second dose in the evening at bed time together with about 100 ml of plain warm water

Statistical analyses The analyses (H SCHOENT, cand. rer nat., Data Processing Center, University of Hamburg) were done as follows Kendall product moment correlation coefficients (r), as well as Spearman rank correlation coefficients (r_s) [21], were computed in the groups under consideration and in large groups composed differently to study the problem of heterogeneity in correlation analyses. The confidence intervals ($p<0.05$) of the correlation coefficients have been read from tables To save space, the results of regression and factor analyses have been omitted. For comparison of the arithmetic means in different test groups (tables II and III), the nonparametric Mann-Whitney U test [21] was used

Results

The results of correlation analyses are shown in table I The null hypothesis that no correlations exists could be rejected at a level of $p<0.001$, when r or r_s values higher than ± 0.34 in the first column and higher than ± 0.26 in the second column were calculated. Despite the low number of paired observations, the degree of association between absorption of 50 mg administered in solution and 50 mg of quick release iron is statistically significant ($p<0.01$)

The data bodies of the present (table I) previous [8] and in the meantime further extended studies differ considerably in number of subjects examined ranges of disease and proportion of iron replete and iron-deficient subjects. Despite of these important heterogeneities high correlation coefficients have been computed between the diffuse Prussian blue reaction of bone marrow macrophages and intestinal iron absorption in nearly every grouping containing divergent proportions of iron replete and iron deficient individuals. The highest values have been obtained with 0.56-mg test doses in the entire population under consideration (n = 927) independent of whether 114 patients with Hodgkin's disease and 49 cases

Table I Correlation coefficients

Clinical variables	Intestinal iron absorption, 50 mg Fe^{++}			
	in solution (n = 103)		in quick-release capsules (n = 146)	
	r	CI	r	CI
Diffuse Fe	−0.66±0.13		−0.48±0.14	
Polymorphous Fe	−0.47±0.18		−0.29±0.15	
0.56 mg Fe absorption	+0.73±0.11		+0.47±0.14	
TIBC	+0.41±0.19		+0.38±0.15	
UIBC	+0.48±0.17		+0.38±0.15	
Plasma iron	−0.28±0.20		−0.23±0.15	
Transferrin saturation	−0.34±0.20		−0.29±0.15	
Sideroblasts	−0.23±0.20		−0.35±0.15	
Haemoglobin	−0.31±0.20		−0.33±0.15	
Haematocrit	−0.23±0.20		−0.28±0.15	
Red blood cells	−0.15±0.21		−0.07±0.16	
MCH	−0.32±0.20		−0.39±0.15	
MCV	−0.24±0.20		−0.35±0.15	
50 mg Fe^{++} absorption (solution), n = 20			+0.82	
	r_s		r_s	
Diffuse Fe	−0.73±0.11		−0.56±0.13	
Polymorphous Fe	−0.63±0.14		−0.35±0.15	

r = Product moment correlation coefficient, r_s = Spearman rank correlation coefficient, CI = confidence intervals of the correlation coefficients ($p < 0.05$)

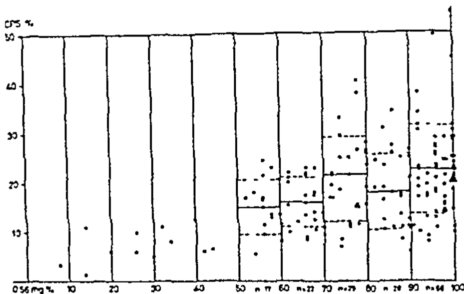


Fig. 1. Bivariate scattergram of intestinal $^{59}Fe^{++}$ absorption (%). 0.56-mg dose (solution). 50-mg dose (quick release iron capsule Hoechst). 164 cases, iron replete individuals and subjects with prelatent, latent and manifest iron deficiency. ——— Arithmetic mean, - - - - Standard deviation.

(table II, fig. 2). The differences between the iron-deficient groups were not significant ($p > 0.05$). The absorption of 50 mg in quick release capsules increased slightly with the severity of anaemia (table II), but the number of subjects with haemoglobin < 6.0 g% was too small for exact comparisons. Divergent correlation coefficients between haemoglobin and 0.56-mg absorption (table III, B) or 50-mg absorption (table I) are probably the product of heterogenous groupings.

Table III and figure 3 demonstrate breakfast inhibition of 0.56-mg absorption in iron replete and iron-deficient subjects who admitted to have had food, coffee, chocolate and/or milk before or soon after administration of the test dose contrary to the advice to remain fasting, but they affirmed that they had fasted during the second examination, showing higher values.

The patients of table III, B, and figure 3 (right side), assumed to be fasting, received the first test dose 1–2 days after cessation of oral iron supplementation with quick release iron capsules (11 individuals) or con-

Table II Intestinal iron absorption of 50-mg doses in fasting subjects

Subjects	n	AM, %	SD, ± %	Range, %
A 50 mg in solution				
Iron replete controls	27	6 7	3 6	0 7–15 0
Prelatent iron deficiency	26	14 4	6 9	2 1–31 5
Latent iron deficiency	10	20 0	8 3	6 0–29 5
Manifest iron deficiency	13	20 5	8 5	11 0–38 0
B 50 mg in quick release iron capsules				
Iron replete controls	16	9 4	5 4	1 5–20 0
Prelatent iron deficiency	68	18 6	7 7	8 5–49 5
Latent iron deficiency	46	18 2	7 0	5 5–38 0
Manifest iron deficiency	45	23 1	9 0	10 0–54 0
Hb 10 0–11 9 g%	25	21 0	8 6	10 0–44 8
Hb 8 0–9 9 g%	10	22 8	5 6	12 8–30.6
Hb 6 0–7 9 g%	7	24.9	15 6	15 4–54 0
Hb <6 0 g%	3	27 4	7 5	19 4–34 3

n = Number of subjects examined AM = Arithmetic mean of iron absorption values SD = Standard deviation The differences between iron replete and iron-deficient subjects in A and B are statistically significant (p<0 01, p<0 05 iron replete controls–prelatent iron deficiency in A)

ventional iron preparations (2 individuals), and the second test dose with higher findings after 3 weeks or later Only one subject with the lowest value in the 0 56-mg series had repleted iron stores (diffuse iron ++, coarse haemosiderin particles +) after treatment with quick-release iron for 6 months up to a total dose of 18 0 g In this group, 2 patients remained anaemic (10 7 g% and 10 2 g% Hb) and 2 further subjects in prelatent iron deficiency despite 6 0 g of quick-release iron during 2 months of supplementation (100 mg of ferrous iron daily) In the 50-mg series, 6 patients showed latent iron deficiency, 1 case was still anaemic (10 4 g% Hb) during the first test, and the last one had prelatent iron deficiency The total doses of antecedent iron supplementation varied from 3 0 g (3 cases) and 6 0 g (3 cases) to 12 0 g (2 cases) The iron stores were not repleted (diffuse iron negative or traces, 0 56 mg iron absorption >70%) Except for one pregnant woman and one female with posthaemorrhagic iron deficiency due to transient thrombocytopenia, all patients of table III, B, and figure 3 (right side) had chronic blood loss

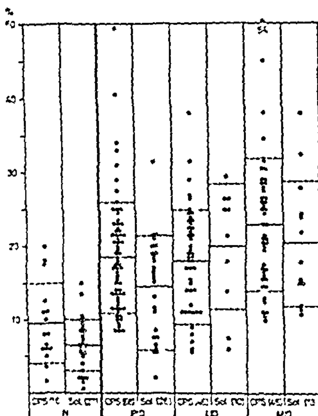


Fig. 2. Intestinal [59]Fe[++] absorption %. ● 50-mg dose (quick-release capsule, *Hoechst*). ○ 50-mg dose (solution). (..) Number of subjects. —— Arithmetic mean. - - - - - Standard deviation. N = Iron-replete subjects. PID = Prelatent iron deficiency. LID = latent iron deficiency. MID = Manifest iron deficiency.

22 (12%) of 179 patients taking one capsule of quick release iron in the morning, 30–60 min before breakfast and a second dose in the evening at bedtime, complained of side reactions, particularly of nausea and epigastric pain. Heavy intolerance occurred in 3 cases. A small double-blind study was performed in 14 patients who had already exhibited side reactions to conventional iron preparations. The standard quick-release iron capsules containing 84 mg of $NaHCO_3$ were tolerated well by 10 subjects, the corresponding capsule without $NaHCO_3$ only by 4 individu-

Table III Section A shows effect of antecedent oral iron supplementation on intestinal iron absorption (first 0 56- and 50-mg test doses given 1–2 days after cessation of therapy, second doses later than 3 weeks Section B shows inhibiting effect of unallowed break fast on absorption of 0 56 mg of iron

	n	AM %	SD±,%	Range %
A Preinhibition by antecedent therapy				
0 56-mg dose				
Preinhibited (1)	5	28 8	28 9	1 8–52.0
Uninhibited (2)	5	63 3	27 7	19 9–86 7
50 mg dose				
Preinhibited (1)	8	8 5	3 6	1 0–12.4
Uninhibited (2)	8	26 2	8 9	10 7–38.2
B Alimentary inhibition on 0 56 mg absorption				
ron replete controls				
Not fasting	8	7 3	6 3	2.2–18 0
Fasting	8	26 3	13 1	9 5–44 4
Iron-deficient subjects				
Not fasting	40	32.4	16 0	4 5–58 0
Fasting	40	71 4	16 1	52.0–98 0

n = Number of subjects examined AM = Arithmetic mean of intestinal iron absorption SD = Standard deviation Differences of paired tests $p < 0\,01$ Each subject served as his own control

als Capsules taken at bedtime caused less side reactions than the morning dose

In vitro tests showed that the quick release iron capsules disintegrate within 2–3 min in warm water, acid and neutral gastric juice at 37 °C with development of CO_2 bubbles and the violet colour of iron ascorbate chelate Conventional iron tablets dissolved much slower Different slow release preparations demonstrated high variation in release of their iron contents

Discussion

The findings reported (table I) indicate that higher degrees of association exist between the diffuse Prussian blue reaction of bone marrow macrophages, 0 56-mg absorption and 50 mg absorption in comparison to the other variables of iron status, independent of divergent proportions of

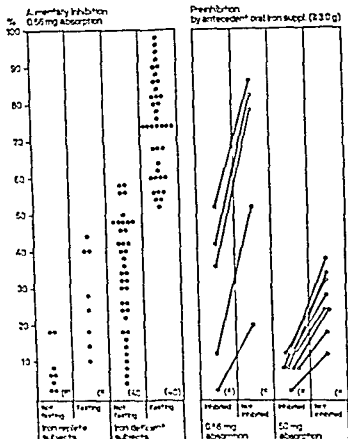


Fig. 3. Intestinal $^{59}Fe^{++}$ absorption. Effect of alimentary inhibition on 0.56-mg absorption and antecedent oral iron supplementation on 0.56- and 50-mg absorption.

iron replete and iron-deficient subjects in the data body. When the test doses were raised from 0.56 mg to 50 mg of ferrous iron, given in solution of 0.01 N HCl or in quick release capsules, the variability of the results increased considerably, as shown by lower correlation coefficients (table I), as well as by the scattergrams of 50-mg absorption (fig. 2). No clear-cut dividing line between iron replete and iron-deficient subjects, which was obvious in previous studies with 0.56-mg test doses (9, fig. 1),

can be found in figure 2 A high degree of variability in the iron deficient range is observed also in the bivariate scattergram of 0 56- and 50 mg (quick-release iron) absorption (fig 1) Further studies are necessary to clarify whether small amounts of ferrous iron are absorbed by an active, carrier mediated process and therapeutic doses by diffusion, as inferred from experiments in mice [5] Other theoretical aspects of ferrous iron absorption in fasting subjects, summarized under the term 'mucosal feedback regulation' have been discussed previously [8] With respect to the therapeutic value of quick-release iron capsules, it is important to emphasize that no significant differences exist between the average absorptions of 50 mg doses from solutions and quick-release capsules Ascorbate stabilizes ferrous sulphate in the bivalent state In fasting ^{59}Fe absorption whole body retention tests [15], ascorbate, succinate, fumarate, and diasparatate did not increase the absorption of ferrous iron given in 50 mg doses

Transient decrease of intestinal ferrous iron absorption by antecedent large iron doses saturating the absorption mechanism partially has been reported to develop after ingestion of single doses [3] and in the course of iron supplementation, when 30–220 mg of ferrous iron was given [6, 7, 16, 17] This effect lasted 1–3 days after cessation of iron intake [16, 17] After treatment with 100 mg of ferrous iron daily, given in quick release iron capsules up to total doses of 3–10 g, similar results (fig 3, table III) have been observed in a few cases with slight anaemia, latent or prelatent iron deficiency before the iron stores in the bone marrow were replenished More studies in iron deficient subjects from severe anaemia to the prelatent stage are required to estimate the development, the magnitude and duration of 'preinhibition' of intestinal ferrous iron absorption during therapy more precisely Seven hours after administration of 200 mg of ferrous iron to patients with hypochromic iron deficiency anaemia, a positive Prussian blue staining of subepithelial macrophages of small intestinal mucosa, thought to act as transitory iron stores, has been observed [1] Similar results have been obtained 12 h after application of 100 mg of quick release iron daily for 3 days in a case of latent iron deficiency [unpublished data] Obviously, in states of 'mucosal preinhibition' due to antecedent iron doses, the RES iron in the bone marrow does not reflect intestinal iron stores effective in inhibition of intestinal iron absorption, as is assumed to be the case in conditions of 'feedback regulation', which represents the behaviour of small intestinal mucosa to low amounts of ferrous iron [8]

Breakfast inhibition of 0.56 mg of ferrous iron absorption (fig. 3, left side, table III) is probably due to dietary constituents and secretory factors [4] rendering a considerable part of ferrous iron administered for diagnostic purposes less available for absorption in iron replete and iron-deficient subjects. Corresponding results after a standard meal have been obtained earlier with radioiron absorption erythrocyte incorporation tests (5-mg dose) [20] and a double isotope technique (36-mg dose) [2, 6]. Immediately after meals, the inhibiting effect has been assumed to be 40% higher than between meals [2]. The absorption of haemoglobin iron has not been decreased by food [22].

By the administration of quick release iron capsules 30–60 min before breakfast and in the evening at bedtime, alimentary inhibition is circumvented as far as possible. The disadvantages of liquid iron preparations causing metallic taste and discoloration of teeth with prolonged use are avoided. Several factors seem to contribute to the low frequency of side reactions: rather low single doses, rapid disintegration of the capsules within the empty stomach supported by CO_2 liberated from $NaHCO_3$ by ascorbic acid, dilution of ferrous iron by plain warm water taken immediately after intake of the capsule, only short exposure of the gastric mucosa to ferrous iron followed by rapid transport of the fluid into the absorbing duodenum and jejunum.

Alimentary inhibition of ferrous iron absorption can be overcome by high doses of ferrous iron given in conventional iron tablets or sustained release capsules taken immediately before, with or after meals, as suggested by therapeutic trials [6, 7, 18, 19]. It remains to be clarified whether and to what extent large amounts of so-called absorption promotors protect ferrous iron from the adverse effect of dietary constituents in the intestinal tract. In evaluation of the potentials of oral iron supplementation, the initial intersubject variation of therapeutic ferrous iron absorption, as shown in figures 1 and 2, should be taken into consideration. The knowledge of preinhibition by antecedent iron doses (fig. 3, table III) [3, 6, 7, 16, 17] and alimentary inhibition (fig. 3, table III) [2, 4, 6, 20] in relation to the iron preparations prescribed to be taken apart from, before, with or after meals, the composition of meals and different digestive phases is still inadequate. Other problems dealt with in two subsequent papers [11, 12] concern the absence and presence of blood loss in the course of iron substitution.

Acknowledgements. We thank Prof. H. C. Heinrich and his coworkers (Division of Medical Biochemistry, Institute of Physiological Chemistry, University of Ham-

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The Histochemistry of Fibrinolysis in Haemophilic Synovial Membranes

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Abstract Haemophilic synovial membranes obtained by synovectomy have been histochemically studied for the localisation of plasminogen activator In the two histological types of synovial membrane (vascular and fibrous type) different degrees of fibrinolysis were detected Higher fibrinolytic activity was found in the vascular type of haemophilic synovial membrane as compared to the normal synovial tissues Activity was low or absent in the synovial membrane undergoing a process of fibrosis These results may help to explain the mechanism of bleeding in haemophilic joints

Key Words
Fibrinolysis
Haemophilic joint
Synovectomy
Synovial membrane

As they are almost totally devoid of thromboplastic activity, synovial membranes depend for local haemostasis entirely on plasma thromboplastin When the formation of plasma thromboplastin is impaired, as in haemophilia, synovial membranes are especially prone to bleeding According to Astrup and Sjölin [1] this is the reason for the relapsing haemorrhages in haemophilic joints

Removal of fibrin from the joints is probably by local plasminogen activator, which has been shown to be confined to the venules and the capillaries of the stromal layer of synovial membranes [3, 5, 11] Stromal proliferation of haemophilic synovial membranes following repeated haemorrhages can be expected to worsen the haemostasis of the joint in these patients because of the increased content of fibrinolytically active tissue The present study is concerned with the histochemical localisation and assay of fibrinolytic activity in haemophilic synovial membranes obtained by synovectomy [9, 10]

Materials and Methods

Specimens of synovial membranes were obtained from the knee joints of 11 haemophilic patients operated for synovectomy according to STORTI *et al* [10]. There were 10 cases of haemophilia A and 1 case of haemophilia B, all of severe grade, 9-27 years old. The samples were taken from the right knee joint in 8 cases and from the left in 3 cases. Synovial membranes from a newborn infant and from 2 patients aged 6 and 18 years, who died without abnormalities of the clotting factors, were used as controls.

The material was quickly frozen with carbon dioxide and stored at -20 °C for periods ranging from 1 to 10 days. The localisation of the plasminogen activator was determined by a modified [6] fibrin slide technique [12].

Sections of 10 μm thickness were obtained on a cryostatic microtome. The sections were collected on coverslips, dried at room temperature for 10 min and spread with 10 μl of thrombin solution (Topostasin, 20 NIH U/ml unbuffered saline) and with 60 μl of 1.0% plasminogen-rich fibrinogen in Sorensen buffer 1/15 M pH 7.8. Plasminogen-free fibrinogen, prepared according to BISHOP *et al* [4] was also used.

The solutions were mixed with a glass rod and evenly spread over the cover slip (24×32 mm). The slides were then left in a moist chamber for 30 min at room temperature to obtain stabilization of the clot.

All slides obtained from each specimen were incubated in a moist chamber at 37 °C for times ranging from 5 to 40 min. After incubation the sections were fixed with 10% formalin vapours, stained with Giemsa and mounted with glycerol jelly.

To detect the presence of non-specific fibrinolytic activity, controls were prepared, as follows: (a) sections covered with plasminogen-free fibrinogen; (b) sections collected on glass slides covered with films of plasminogen-rich fibrinogen previously heated to 80 °C for 30 min to inactivate plasminogen; (c) sections covered with plasminogen-rich fibrinogen, fixed in formalin vapours before incubation; (d) sections covered with plasminogen-rich fibrinogen and ε-aminocaproic acid (EACA) 2.8 mg/100 μl. The latter controls were incubated and processed in the same way as the sections covered with the plasminogen-rich fibrinogen.

Samples from haemophilic and normal synovial membranes were also fixed in Bouin's fluid or formalin 10%, processed according to conventional histological methods and stained with HE, Van Gieson's, Weigert's and Perls' methods.

Results

Histology

In general we observed two main histological types of haemophilic synovial membranes, a vascular type, rich in blood vessels, and a fibrous type where blood vessels were almost or totally absent. In the vascular type, the synovial internal layer was thickened, often showing a positive Prussian blue reaction.

The presence and organisation of small fibrin clots on or under the intimal lining was seldom noted (fig 1) In the subintimal[1] tissue various degrees of cellular inflammatory infiltration could be seen (lymphocytes, plasma cells and mastocytes) Round cells were seen evenly distributed or grouped around blood vessels Very often haemosiderin granules, histiocytes containing haemosiderin and agglomerations of erythrocytes could be observed in the interstitial tissue Moreover, we noted a marked increase in the size and number of capillaries and venules lying beneath the intimal layer and in the deeper stromal tissues Newly formed capillaries exhibited either a thin endothelial layer or a thickened endothelium with pericapillary hyaline sclerosis Owing to the presence of dilated venules, the stromal tissue sometimes assumed the appearance of an angiomatous tissue

The fibrous type, referred to above, was characterised by the presence of fibrin on the intimal layer to a greater extent than was observed in the case of the vascular type, with evidence of focal fibrin organisation in the form of hyaline deposits, frequently with disappearance of the intimal layer (fig 2)

The subintimal and stromal tissues consisted of very dense collagen tissue with only a residual vascular component These changes most frequently were observed in the non villous areas of the synovial membranes Both the histological types described could co exist in the same synovial membrane

Histochemistry

In *normal synovial membranes*, approximately 10 min was the shortest incubation time for producing areas of lysis Fibrinolytic activity was present ... foci aro... capillaries and venules of the connective tis... the int... In most cases the lytic activity observed ... of th... lining was seen to or... nate in the ves... ent of syn... cells occurred ... these ... seen to pr... fibrinolysis A p... of ... caused co... zones of lysis in the ...

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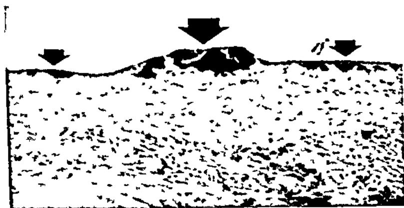




Fig. 1. Fibrin deposits along the synovial surface, which appears devoid of intimal cells. Weigert [illegible]

Fig. 2. Haemophilic synovial membrane evolving towards fibrosis. The intimal layer has disappeared; dilated vessels can be observed in the subsynovial tissue. HE [illegible]

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Histochemistry

In *normal synovial membranes*, approximately 10 min was the shortest incubation time for producing areas of lysis Fibrinolytic activity was present as scattered foci around the capillaries and venules of the connective tissue and along the intimal layer In most cases the lytic activity observed on the surface of the synovial lining was seen to originate in the vessels of the subintimal layer When detachment of synovial cells occurred during sectioning, these cells were often seen to produce fibrinolysis A period of incubation of 30 min generally caused confluent zones of lysis in the subintimal and stromal layers

Fibrinolysis appeared in the vascular type of *haemophilic synovial membrane* after 5 min incubation Lytic areas, rarely observed along the

[1] 'Subintimal' refers to the area immediately beneath the synovial 'intimal' cells Stromal capsular layers are indicated as subsynovial tissues

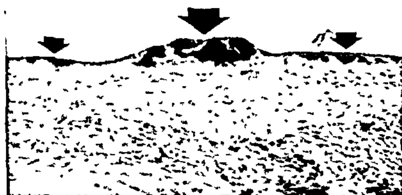


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Fig. 4. Haemophilic synovial membrane. Fibrinolysis has taken place in all the section but in one area (arrow). Histochemistry, fibrin slide technique, incubation time 30 min. × 12.

Fibrinolysis appeared in the fibrous areas of the synovial membranes after about 10 min incubation. There were no areas of lysis along the synovial lining, but these were localised around some stromal capillaries, although not evident until after 30 min incubation (fig. 10). When using plasminogen free fibrin, no lytic activity was observed before 40 min incubation. Fibrinolytic activity was not observed at any time of incubation when using fibrin slides pre-heated to 80 °C for 30 min, fibrin slides fixed with formalin before incubation, or slides with fibrin containing EACA.

Discussion

According to our results, the lesions most frequently found in haemophilic arthropathy are mild to severe haemosiderosis and fibrosis of the



Fig. 8. Higher magnification of the superficial layers represented in figure 7. Lysis is present along the intimal, subintimal and subsynovial tissues. × 45.

tributions in relation to the different pathological features of the tissue. Clear evidence of fibrinolysis along the intimal layer has never been obtained either in normal or haemophilic synovial membranes. When present, lysis appeared to be related to the vessels of the subintimal vascular tissue. However, active sites did appear at areas of cellular dislocation. It is known [8] that the fibrin slide technique produces evidence of a class of cells that require trauma or disintegration for the liberation of the plasminogen activator.

The presence of isolated fibrin clots along the intimal layer suggests that, because of low or absent plasminogen activator activity, these structures are unable to dissolve fibrin. When highly vascular granulation tissue is present in the synovial membranes, lysis is often seen after only 5 min incubation.

Until they became hyalinized or atrophic vessels of scar tissue, the blood vessels of the granulation tissue provided a rich source of tissue

9



10

Fig. 9. Higher magnification of the subsynovial tissues represented in Fig. 7. Lysis is evident around proliferating capillaries of the granulation tissue and around dilated vessels. Histochemistry. Fibrin slide technique. Incubation time 30 min. × 250.

Fig. 10. Fibrinolytic area similar to that of Fig. 2, demonstrating fibrin digestion in relation to the dilated vascular spaces. Histochemistry. Fibrin slide technique. Incubation time 30 min. × 250.

plasminogen activator activity This finding is in agreement with previous results [2] No activity has ever been seen at the site of inflammatory cells KWAAN and ASTRUP [7] obtained a lysis related to phagocytes, but they used a longer incubation time (60 min) They regarded the result as consistent with non-specific proteolytic activity

It might be thought that the high fibrinolytic activity often observed in haemophilic synovial membranes contributes to further disturbances of the local haemostasis of the joints in haemophiliacs In our opinion, in these patients the strong fibrinolytic activity of the haemophilic synovial membranes, coupled with a deficient plasma thromboplastin system, renders these tissues particularly prone to bleeding Quite a different situation exists in synovial membranes of fibrous type and in fibrous scar tissue *The low content of plasminogen activator in the fibrous* areas tends to cause deposition of fibrin, a thickening of capsule without evidence of granulation tissue and a delay in tissue repair

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Acta Haemat 49 154–158 (1973)

Diagnosis of Meningeal Leukemia in Acute Childhood Lymphocytic Leukemia with Periodic Acid-Schiff Reaction of Cytocentrifuged Liquor

A J Feldges

St Jude Children s Research Hospital Memphis Tenn

Abstract The cerebrospinal fluid (CSF) of 22 consecutive untreated children with acute lymphocytic leukemia who entered total study VII from September to December 1971 at St Jude Children s Research Hospital was examined Four patients had periodic acid Schiff (PAS) positive Sudan black B and peroxidase negative lymphoblasts in the CSF at diagnosis Two of the 4 developed meningeal leukemia 3 and 5 months following diagnosis None revealed pleocytosis and/or detectable atypical cells on a Wright stained smear of CSF sediment at diagnosis Two patients had no more detectable PAS positive lymphoblasts after prophylactic central nervous system (CNS) radiotherapy They are in continuous complete remission for 9 and 10 months The recognition of a small number of CSF abnormal cells with PAS reaction and the differentiation of blasts from inflammatory cells may allow an earlier diagnosis and treatment of CNS involvement in childhood acute lymphocytic leukemia

Key Words
Acute lymphocytic leukemia
Childhood leukemia
Meningeal leukemia
PAS reaction

Central nervous system (CNS) leukemia is diagnosed when 2 or more leukemic blast cells are found in the Wright stained centrifugate of 2- to 5 ml sample of spinal fluid Routine lumbar punctures have revealed the presence of leukemic cells even in the absence of signs or symptoms of CNS leukemia [4, 10, 14] In one study, 41% of cerebrospinal fluid (CSF) samples with routine cell count of 4 or less cells revealed some immature cells on cytology [9] However, often when CSF cells are less than 20/mm³, the morphology of the atypical cells may be questionable and therefore a second sample of spinal fluid is required for diagnosis of meningeal leukemia This paper reports the value of periodic acid

Schiff (PAS) staining of centrifuged spinal fluid for the identification of lymphoblasts and the differentiation from other mononuclear cells that are often mistaken as leukemic cells.

Methods

The CSF of 22 untreated children who consecutively entered total study VII (protocol VII) from August 27 1971 to January 1 1972 at St Jude Children's Research Hospital was examined cytochemically with Sudan black B staining, PAS and peroxidase reaction. Samples for cytochemical examination were obtained at diagnosis after prophylactic CNS radiotherapy and routinely every 12 weeks.

PAS and peroxidase reaction were used as recommended by Hayhoe [6], Sudan black B by the technique of Sheehan and Storey [13].

Preparation of CSF sediment: 2 ml CSF for each staining technique was centrifuged in a Shandon cytocentrifuge at 1,000 rpm for 3 min. The sediment was removed, air-dried, fixed and immediately stained.

Results

In 4 of the 22 patients PAS positive lymphoblasts could be identified in the CSF at diagnosis. None revealed pleocytosis or detectable atypical cells on a Wright stained liquor sample. Sudan black B staining and peroxidase reaction were negative. The PAS reaction exhibited a moderate to intensive reactivity. The nature of the positivity was one of coarse granularity or block deposits against a clear background of the cytoplasma.

The clinical follow up studies and the results of repeated lumbar punctures of the 4 patients with PAS positive cells in the CSF are given in table I.

Following 4 to 5 weeks of remission induction therapy with prednisone and vincristine, 12 of the 22 children were randomized to receive 2,400 rad cranial irradiation plus twice weekly intrathecal methotrexate and 10 children were randomized to receive 2,400 rad craniospinal axis irradiation.

Of the 12 patients given cranial irradiation with intrathecal methotrexate, 3 (25%) developed mild pleocytosis (11–17 WBC/mm³). None of the 3 patients had detectable lymphoblasts on Wright or PAS staining in the centrifuged liquor.

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CNS relapse, compared to 27 of 49 patients (55%) who did not receive this form of therapy [1, 2].

Irradiation of the craniospinal axis with 2400 rad must thus destroy clinically undetectable leukemic cells in the CNS. To identify lymphoblasts in the CSF at diagnosis with more accuracy, PAS staining of the cytocentrifuged liquor was performed. Of 22 patients with no detectable lymphoblasts in Wright-stained liquor, 4 showed PAS positive lymphoblasts in their CSF at diagnosis. Two of these 4 patients had PAS-positive lymphoblasts still after prophylactic CNS radiotherapy. They both developed meningeal leukemia with typical signs and symptoms later in the course of their disease.

We conclude from these data that PAS staining of the cytocentrifuged liquor is a sensitive method for the identification of lymphoblasts in the CSF.

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Ready Release of Intracellular Muramidase (Lysozyme) from Mononuclear Cells in the Skin Window Exudates

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Abstract. Muramidase, acid phosphatase and nonspecific esterase were investigated in mononuclear cells of skin window exudate in normal persons and patients with various forms of leukemia. While acid phosphatase and nonspecific esterase gradually increased, muramidase activity was reduced. Fifty hours after the onset of the experiment, most of the mononuclear cells were muramidase negative. In monocytic leukemia, mononuclear cells appeared rapidly in the exudate. The ready liberation of muramidase may play a significant role for the increased muramidase in this type of leukemia.

Key Words
Acid phosphatase
Cytochemistry
Esterases
Leukemia
Monocytes
Muramidase
Skin window technique

Markedly elevated serum and urine muramidase (lysozyme) in patients with monocytic leukemia [2, 6, 10, 13–16, 24, 25] has been attributed to the increase of monocytic cells. Proliferation of granulocytic cells, especially of mature ones as in the case of chronic myelocytic leukemia also causes an increase of serum and urine muramidase, though to a lesser extent than that in monocytic leukemia.

The present study was undertaken to investigate the change of activities of muramidase and other hydrolytic enzymes in cells appearing in skin window exudates of normal persons and patients with monocytic leukemia and other types of leukemia in an attempt to clarify the mechanism of increased quantity of muramidase in monocytic leukemia.

Materials and Methods

Skin window studies were carried out following the method of Rebuck and Crowley [17] on 6 healthy persons, 5 patients with monocytic leukemia, 4 with

blood monocytes and macrophages in other sites such as bone marrow, pleural and peritoneal cavity which usually lack the enzyme

Rapid and cellular exudation of monocytes in tissues, and the ready liberation of muramidase from monocytes than from neutrophils may contribute to the increase of muramidase in monocytic leukemia

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Recombination Experiments with an Unusual L-Chain Related Myeloma Protein[1]

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Abstract The occurrence of a light chain related myeloma protein, which was defective compared with complete light chains gave rise to further investigation of its structure Mixing of ^{125}I labelled polypeptide chains resulting from the reduction of this protein with heavy and light polypeptide chains of an ^{131}I labelled myeloma protein showed recombination to a considerable extent Fragments of L chains as it appears from these experiments, may exist as dimers stabilized by noncovalent bonds Their interaction with light and heavy chains *in vivo* might compete with the normal assembly of immunoglobulin polypeptide chains.

Key Words
Antibody formation
Immunoglobulin structure
Light chain fragments
Myeloma proteins
Polypeptide chain

The regulatory mechanisms, which are working in antibody synthesis are unknown One of the main characteristics of the immunological response is its self-limiting character The myeloma cell has lost this control for an obscure reason and produces immunoglobulins in large excess, leading to the accumulation of a homogeneous species of cells and their products, the so-called paraproteins, in the serum of myeloma patients These genetically uniform proteins proved to be very worthful for the elucidation of the mechanisms responsible for the antibody variability Myeloma proteins are generally accepted as a model of antibody molecules, whose specificity is reflected by the variability of amino acid sequences of individual myeloma proteins It is of interest that this vari-

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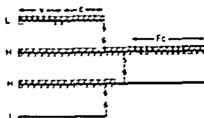


Fig. 1. Schematic presentation of polypeptide chains and fragments (hatched respectively cross hatched area) of the IgG molecule found in the serum and/or urine under normal and/or pathological conditions. The length of the H-chain fragments is arbitrarily.

ability pertains only to a part of the heavy (H) and light (L) chain, which form the antibody molecule (fig. 1). Those parts of the γ-globulin molecule, which are marked by hatched or cross hatched zones have been observed in the serum and/or in the urine as fragments under normal or pathological conditions [3, 4, 9, 10, 14, 18, 24].

Our experiments are concerned with a protein which was isolated from the serum and the urine of a myeloma patient (Fe.) and which, according to its 'fingerprints' represents fragments of L-chains, probably related to the V half of an L-chain [19]. The myeloma protein Fe. consists of two kinds of polypeptides, called fraction I and fraction II protein after their chromatographic behavior.

The occurrence of those fragments in the urine rises the question, if they are degradation products or if they are biosynthetic units. From the behavior of L-chain fragments during the treatment by an alkylating agent, we concluded that these proteins stem from biosynthesis and not from degradation [19].

With this finding several questions arise. Are there two populations of myeloma cells, synthesizing the fraction I and fraction II protein? Why is the occurrence of fragments of L-chains such a rare event? Out of 260 cases with myeloma proteins the observation of this kind was unique. And further the question arises, why the myeloma cells in some patients do not secrete their paraproteins but accumulate them intracellularly. Which are the structural conditions for myeloma proteins being transferred through the cell membrane?

Considering the fact, that in the vast majority of myeloma cases complete γ-globulin molecules are found, it seems probable that normally

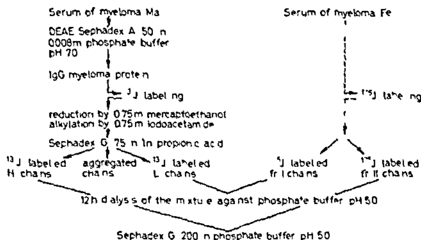


Fig 2 Procedure of preparation and reduction of myeloma proteins and separation of their constituent polypeptide chains for recombination

the combination of H- and L-chains of such molecules is a preliminary condition for permeation of the cell membrane This assumption is supported by the finding, that normally less than 1% of the total synthesized amount of L-chains are excreted [7]

Regarding these questions it seemed of interest, to study the behavior of the above-mentioned myeloma protein Fe under recombination conditions of its constituent polypeptide chains The experimental procedure is shown schematically in figure 2 The principle is that ^{125}I labelled 'fr I-chains' and 'fr II-chains' are mixed with ^{131}I-labelled H- and L-chains from an IgG-myeloma protein Further experimental details have been communicated previously [20]

Materials and Methods

Myeloma protein Ma has been isolated from the serum of a patient Ma by chromatography on DEAE Sephadex A 50 It has been identified as an IgG protein of light chain type λ Details of the protein Fe have been mentioned above and communicated elsewhere [21] This protein shares antigenic determinants with λ-chains Iodination of myeloma protein Fe and Ma was performed according to McCONAHEY and DIXON [11]

NaJ^{131} and NaJ^{125} was obtained from Hoechst Frankfurt The specific activity was 100-200 μCi/mg protein The counting was performed in a Siemens/Telefunken

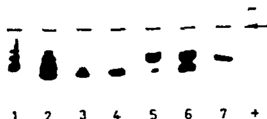


Fig. 3. Starch gel electrophoresis of fr. I(1), fr. II(3) of the reduced alkylated ^{131}I labelled myeloma protein Le (2) and fr. III(4), fr. III(5), fr. I(7) of the reduced alkylated ^{131}I labelled myeloma protein Ma (6) after gel filtration on Sephadex G-75.

automatic liquid scintillation counter. Immunoelectrophoresis was performed according to SCHEIDEGGER [15]. Commercial antisera from the Behring Werke, Marburg, and antisera made in rabbits by immunization with protein Le have been used. Starch gel electrophoresis was performed according to SMITHIES [13]. Sedimentation analyses were done at 20 °C in the analytical ultracentrifuge Spinco Model E. For the reduction, alkylation and gel filtration of myeloma proteins the procedure given by FLEISCHMAN *et al.* [4] has been followed (fig. 2). For recombination experiments[1] H- and L-polypeptide chains Ma and fr. I and fr. II chains Le were mixed at a ratio of 3:1 on a mass basis.

Results

Figure 3 shows the result of starch gel electrophoresis of myeloma protein Le and Ma. As indicated in figure 2 gel filtration of reduced myeloma protein Ma on Sephadex G-75 in 1 N propionic acid leads to 3 fractions. Fr. I consists of H-chains in an aggregated form, fr. II of H-chains in monomeric or dimeric form and fr. III of L-chains. For the recombination experiment A fr. II chains of the reduced ^{131}I labelled myeloma protein Ma were mixed with fr. II of the reduced ^{131}I labelled myeloma protein Le. As shown in the upper part of figure 4, a considerable amount of labelled fr. II protein Le appears in the elution profile at a position where the recombination product is located (fig. 4, lower part). That is at tube No. 22. The extent of recombination calculated from the dislocation of Le-chain protein is 45% in experiment A

[1] 'Combination experiments' would be a more correct term, because heterologous polypeptide chains were combined.

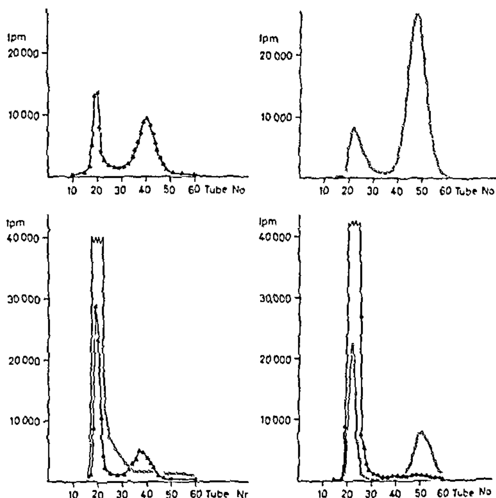


Fig 4 Elution diagram of ¹²⁵I labelled fr II protein Fc (▲) on Sephadex G 200 in Sorensen buffer pH 7 0 (above) and of a mixture of these polypeptides and ¹³¹I labelled H polypeptide chains of IgG myeloma protein Ma (o) in Sorensen buffer pH 5 0 (experiment A)

Fig 5 Elution diagram of ¹³¹I labelled L-chains of IgG myeloma protein Ma on Sephadex G 200 in Sørensen buffer pH 7 0 (above) and of a mixture of these L-polypeptides (o) and ¹²⁵I labelled fr I protein Fc (▲) (experiment B)

Compared with the behavior of protein Fc in figure 4 (upper part) gel filtration of the L-chains of myeloma protein Ma shows only a small fraction in the region of tube No 22 (fig 5, upper part) The recombination amounts to 43% in this experiment (B), that is nearly the same as in experiment A

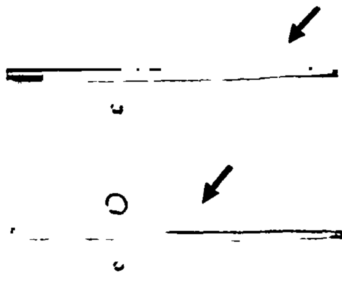


Fig. 6. Immunoelectrophoresis of the recombination product of experiment A developed by an anti-λ antiserum (a) and of experiment B by an anti-κ antiserum (b). The wells of the lower part of each slide contain normal serum (the position of the 'new' proteins is marked by arrows).

The sedimentation analysis of H-chains of myeloma protein Ma. exhibits a strong tendency to aggregation, the monomeric form showing values about 4.6 s [22]. It is of interest, that a positive result of recombination experiments was achieved with aggregated H-chains of 10.3 respectively 11.3 s. Fr. I protein showed a sedimentation coefficient of 6.4 s, the fr. II protein a coefficient of 3.6 s. After recombination of H-chains Ma. and fr. II protein Fc. two components of 8.5 and 5.9 s were observed.

Immunoelectrophoresis of this recombination product developed by an anti-λ antiserum gave a precipitation line, whereas the recombined protein from fr. I protein Fc. and L-chains Ma. reacted with an anti-κ antiserum (fig. 6). As protein Fc. carries λ-specificity and protein Ma. is of light chain type κ, the immunoelectrophoretic behavior of these 'new' proteins follows the expected pattern.

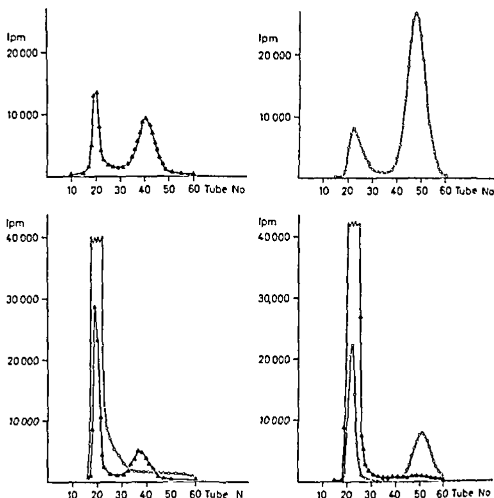


Fig 4 Elution diagram of ^{125}I labelled fr II protein Fc (▲) on Sephadex G 200 in Sørensen buffer pH 7 0 (above) and of a mixture of these polypeptides and ^{131}I labelled H polypeptide chains of IgG myeloma protein Ma (o) in Sørensen buffer pH 5 0 (experiment A)

Fig 5 Elution diagram of ^{131}I labelled L-chains of IgG myeloma protein Ma on Sephadex G 200 in Sørensen buffer pH 7 0 (above) and of a mixture of these L-polypeptides (o) and ^{125}I labelled fr I protein Fc (▲) (experiment B)

Compared with the behavior of protein Fc in figure 4 (upper part) gel filtration of the L chains of myeloma protein Ma shows only a small fraction in the region of tube No 22 (fig 5 upper part) The recombination amounts to 43% in this experiment (B), that is nearly the same as in experiment A

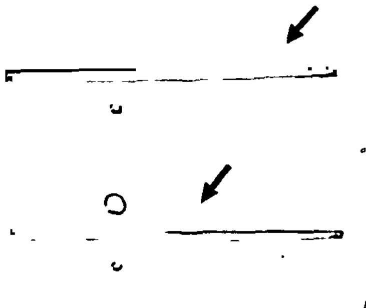


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Discussion

From immunological, electrophoretical and chromatographical studies after tryptic digestion, it appeared that the myeloma protein Fe consists of L-chain related polypeptide chains [21] No heavy chain determinants could be detected by immunoelectrophoresis or immunodiffusion analysis according to Ouchterlony Reduction by mercaptoethanol followed by gel chromatography yielded two fractions, which differed by one peptide L-chain related proteins of a molecular weight between 10,000 and 20,000, that is smaller than intact L-chains, have been found by several authors [6] The question was studied, if those proteins were of anabolic or catabolic origin by means of radio-labelled Bence Jones proteins [5] After reinjection of those proteins into patients, these authors found radioactive fragments of L-chains in the urine of some cases These fragments, which were related to the variable half, exhibited a characteristic sedimentation behavior at neutral and at alkaline pH [2] Uroprotein Fe, which has been used for our experiments, showed these properties Nevertheless, protein Fe cannot originate in the urinary tract, as it is the case with the above-mentioned fragments of Bence Jones proteins, because the same protein could be demonstrated in the serum of this patient [21] The relationship of the L-chain related protein Fe to the v-half of a L-chain was inferred from the sedimentation coefficients at pH 5 0 and 11 0 and from unusual low reactivity with anti-k and anti-λ-antisera, respectively If protein Fe corresponds to the v-half of a L-chain, this reactivity has to be reasoned, because the k and λ common antigenic determinants are supposed to reside in the constant region Other authors [17] had a similar experience with a k-fragment and discuss the structural implications In addition to the points raised in the paper of these authors the possibility has to be taken in account, that the antigenic determinants shared by the v- and c-half might be of conformational character

The elution diagram of the reduced and alkylated protein Fe and of L-chains of protein Ma shows two components, one with its maximum at tube No 40, which is characteristically for L-chains under the experimental conditions and one with its maximum at tube No 20 This latter component consists of aggregated L-chains As to the spatial arrangement of polypeptide chains of protein Fe no conclusion can be drawn from the recombination experiments

From the sedimentation coefficient of the protein Fe of 3 6 s [21],

the occurrence of two sorts of constitutive polypeptide chains (a, b) in the form of dimers as revealed by starch gel electrophoresis [19] and from the separation of these two kinds of dimerized polypeptides after reduction by mercaptoethanol, the structure of protein Fe has been formulated by a a·b b [21] By the different symbols in this formula the presence of noncovalent ( ) and covalent (·) bonds is indicated The absence of disulphide bonds in dimers of L-chains has been shown to be a premise for the recombination with H-chains [16] This finding might apply to L-chain fragments in recombination experiments as well

It remains to be discussed that fr I protein Fe combines with L-chains Ma Complete L-chains of different myeloma proteins do not combine under the conditions observed in these experiments [unpublished results] The question is, if fragments of L-chains, regularly show this behavior

Marked differences between the polypeptides resulting from reduction and alkylation of myeloma protein Fe on the one hand and H- and L-chains of the myeloma protein Ma on the other are visible in figure 3 Whereas H-chains of protein Ma are clearly separated from L-chains, the fr I and fr II protein Fe does not resolve in a comparable manner Moreover it can be seen that fr I protein reveals at least 5 components instead of 2 components found in earlier experiments [21]

The cause for this growing tendency of polymerization is not evident (the proteins were storaged lyophilized at −20 C) The arrangement of electrophoretic components within the fr I protein is in contrast to the H-chain heterogeneity, indicating the structural differences of these proteins From figure 3 it follows that the G 75 fraction II from the reduced myeloma protein Ma is contaminated with L-chains and cannot be used for recombination experiments

With respect to the problems outlined in the introductory remarks the question arises, if from the structural peculiarities of the myeloma protein Fe informations about the disturbances of the underlying cellular or intracellular regulatory processes can be gained From pulse labelling experiments a model of γ-globulin synthesis was developed according to which a pool of free L-chains controls the H-chain synthesis [1] One could speculate that the release of H-chains in the myeloma cells of the patient Fe does not operate normally, because of incomplete new L-chains

As to the question if fr I protein and fr II protein are synthesized in the same cell or in different clones as it was discussed previously [23]

no decision can be made from the results The assumption that two cell clones are involved in the synthesis of protein Fe has been made under the impression of a significant change in the ratio of fr I and fr II protein Fe excreted in the urine with the application of 45 mg *p* di (2 chlorethyl) amino L phenylalanine (Melphalan®) [21] Since it has been found, that Melphalan induces specific changes in the t RNA profile of myeloma cells in mice [12] we might deal with a mutation Recently, it could be demonstrated by immunofluorescence studies in a case of *chronic lymphatic leukemia that fragments of μ-chains and L-chains of* k type were present in the same cell [12]

These authors discuss a mechanism which is the reverse of the one suggested in the case Fe, namely, blocking of chain assembly due to defective H-chain synthesis and secondary accumulation of L chains

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Myeloma in Mother and Daughter

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Abstract The occurrence of multiple myeloma in mother and daughter is described To the best of our knowledge, this has not been previously reported with both family members available for detailed study

Key Words
Cancer and bone marrow plasma cells
Familial myeloma
Myeloma
Plasmacytoma

The occurrence of myeloma in two or more members of one family is rare and only once previously has it been described in parent and offspring [10] We report the diagnosis of IgA myeloma in a 60 year old woman and the finding four years later of IgG myeloma in her 84-year-old mother We believe this to be the first fully substantiated report of the disease occurring in two generations of the same family

Case Histories

Case 1 The daughter a 60-year old woman of Italian extraction was referred to another hospital in 1968 complaining of the symptoms of anaemia occasional dyspepsia and backache There was nothing significant in the past history On examination she was anxious and pale Her blood pressure was 190 100 Otherwise physical examination was normal save for some wasting of the muscles around the shoulder girdle

Haemoglobin 10 0 g 100 ml WBC 5 700/mm³ platelets 200 000 mm³, ESR 67 mm in the first hour (Westergren) Liver function tests, urea and electrolytes were normal Chest spine and skull were X rayed and all showed lytic areas consistent with myeloma There were translucent areas in the pelvic bones Bone marrow examination revealed heavy infiltration by abnormal plasma cells (50% of all cells) (fig 1) The urine was negative for Bence Jones protein on electrophoresis

but there was an abnormal band on serum protein electrophoresis running in the β region which was later characterised as IgA. IgG was reduced.

She remained reasonably well and was treated conservatively. During 1969 and 1970 she developed tenderness over the dorsal spine and sternum. X-ray of the latter showing a fracture. Further X-rays in January 1971 showed a fracture of the left 10th rib and in February 1972, more marked osteolytic areas in the skull and evidence of osteoporosis in the spine and pelvis. There were no collapsed vertebrae. Since diagnosis urea, electrolytes and liver function tests have remained normal. Bence Jones proteinuria has never appeared. Total serum proteins have varied from 7.7 g/100 ml in March 1969 to 8 g/100 ml in January 1972. The most recent electrophoresis in April 1972 showed a persisting IgA band of 3,278 mg/100 ml with 468 mg/100 ml IgG and 34 mg/100 ml IgM. In April 1972, radiotherapy to the sternum was initiated. She has had no other therapy.

This patient therefore suffers from IgA myeloma, is anaemic with a high ESR, has multiple lytic areas in the skeleton, myeloma cells in the bone marrow and a monoclonal IgA band with decreased IgG and IgM.

Case 2. In March 1972, the 84-year-old mother of the previous patient was referred to the Northern General Hospital in Edinburgh with pain and swelling of the left upper arm and inability to move it for approximately one week. She was admitted to hospital. On systematic enquiry, she had a poor appetite and occasional dry cough. She complained of ankle oedema over many years. In 1958 she had had an undifferentiated peripheral carcinoma of the right lung resected. Clinical examination showed dry skin and hair and pallor. There was slight bilateral ankle oedema. The chest showed the scar of the previous right lobectomy. There was no evidence of recurrence of tumor. The left arm was swollen and painful and X-ray showed a mid-shaft fracture of the left humerus with evidence of Paget's disease.

Haemoglobin 10.9 g/100 ml, PCV 3[illegible]%, MCHC 34%, reticulocytes 2[illegible]%, platelets 116,000/mm³, WBC 9,100/mm³ with 52% neutrophils, 40% lymphocytes, 4% monocytes and 4% eosinophils. ESR 66 mm in the first hour. A few macrocytes were seen in the peripheral blood film. Serum vitamin B_{12}, serum folate and Schilling test were normal. Skeletal survey showed patchy decalcification of the skull with a little sclerosis. There was gross decalcification of the lumbar spine and pelvis with collapse of vertebral bodies and a destructive arthritis of the left hip. The upper end of the left humerus showed evidence of previous fracture. The right humerus was markedly demineralised and severely affected by Paget's disease but with no evidence of fracture. In view of the macrocytes in the peripheral film and clinical evidence of pathological fracture, the bone marrow was examined. It was cellular with normal erythropoiesis and granulopoiesis but there were 12% plasma cells, many of which were morphologically pathological with a large single nucleolus. These were considered to be myeloma cells (fig. 2). Iron stores were normal. Biochemistry showed normal electrolytes, blood urea 66 mg/100 ml, creatinine clearance 45 ml/min, and a raised alkaline phosphatase of 352 IU/100 ml consistent with her Paget's disease. Total serum proteins were 7.0 g/100 ml and electrophoresis showed 48% albumin and a monoclonal band. Further characterisation revealed this to be IgG 2,4[illegible] mg/100 ml with IgA of 218 mg/100 ml and IgM of 121 mg/100 ml. There was proteinuria 1[illegible] g/24 h, but Bence Jones protein was not detected [illegible]

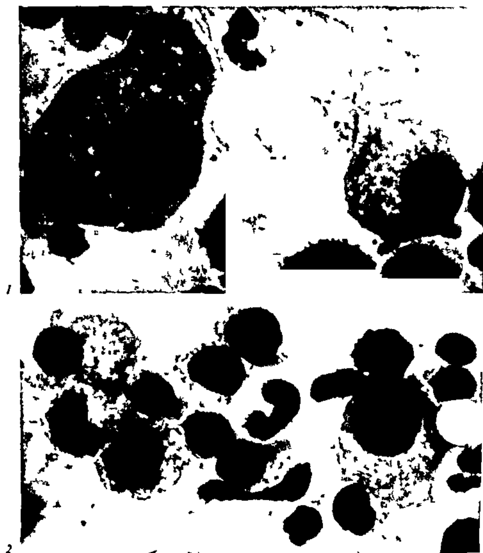


Fig. 1 Bone marrow of case 1
Fig. 2 Bone marrow of case 2

The diagnosis of myeloma therefore was based on the finding of abnormal plasma cells in the bone marrow, a monoclonal IgG serum band and humeral osteolytic lesions. The continued presence of normal levels of the non-affected immunoglobulins in the serum is unusual but is occasionally the case and may be related to the early stage of the disease with as yet a modest quantity of paraprotein. During hospital admission she also spontaneously fractured the right humerus.

Family investigation All available first degree relatives were examined. None showed myeloma or an abnormal paraprotein band. Those examined include 2 brothers and the daughter of our case 1, i.e. two sons and a grand-daughter of our case 2.

Discussion

Familial myeloma is first mentioned in the literature in 1925 when MEYERDING [8] reported a patient with myeloma whose aunt had a pathological fracture of the leg. In 1928, GESCHICKTER and COPELAND [2] cite DARNICK who observed a patient with myeloma whose brother was already suffering from the same disease. These 2 reports lack precise documentation.

There have been other reports of myeloma in siblings. HIRSCH and SCHWARZ [4] reported 2 sisters aged 59 and 57. The first complained of back pain, had osteolytic lesions and an abnormal serum protein electrophoretic strip. The second, with a thickened swollen sternum, had marrow involvement and urinary Bence Jones protein. HERRELL *et al* [3] described 2 brothers both with bone pain. They had lytic changes on bone X rays and gross albuminuria. Urinary Bence Jones was confirmed in one of them only. The 2 sisters reported by MANSON [7] both had backache and X ray evidence of osteoporosis of the spine. Each showed hyperglobulinaemia but no mention is made of the characteristics of the globulin excess. THOMAS [12] treated a brother and a sister. The brother, younger by one year, presented 3 years before his sister with an anaemia for investigation. There were 60% of myeloma cells in his bone marrow and X-rays of the skeleton showed diffuse demineralisation, most marked in the spine, which also showed collapse of vertebral bodies. His sister complained of low back pain. She had abnormal X rays, a monoclonal band running in the β region on protein electrophoresis, bone marrow involvement and had had a malignant breast tumour removed 25 years previously. Of the sisters described by LEONCINI and KORNGOLD [6] one had a serum IgA band and no Bence Jones urinary protein whilst the other had a Bence Jones band only, both in the serum and in the urine.

The only previous report of the disease in 2 generations of the same family is from NADEAU *et al* [10]. They described in detail the disease in brother and sister. Their father had died in the same hospital 27 years previously with osteolytic lesions in the spine, a raised total serum

protein and uraemia Myeloma is the probable diagnosis but the bone marrow was not examined nor was a *post mortem* examination performed

In our case 2, the previous history of a resected undifferentiated carcinoma of the lung, some 13 years before the myeloma was diagnosed, merits comment WEITZEL [13] found at autopsy an incidence of 19 3% of carcinomas in malignant plasma cell dyscrasia There is no evidence of recurrence of tumour in our case and review of the histological sections of the resected specimen has confirmed the diagnosis Patients with carcinoma may have as many as 20% of plasma cells in the marrow [11], although these are 'reactive' cells distinguishable from myeloma cells ANDERSON and VYE [1] have described 53 patients collected from the literature with dysproteinaemia of the myeloma type with associated neoplasm None was a primary lung carcinoma, the commonest sites being colon (in 9 cases) and prostate (in 5)

It is fascinating to speculate about the aetiology and pathogenesis of the disease in these familial cases MITCHELL *et al* [9] have recently provided evidence of possible transmissibility of human IgG myeloma in immunologically deficient mice where, by passage, broken-cell filtrates consistently produced myeloma in marrow and spleen and human immunoglobulin in the serum Work on in-bred strains of mice has shown the importance of genetic and transmissible factors in the aetiology of plasma cell tumours at least in animals The in-bred C_3H mouse strain and the F1 hybrids of CBAx DBA2 mice develop 'spontaneous' plasma cell tumours Moreover, a plasma cell dyscrasia affecting the Aleutian mink is now known to be caused by virus

HOBBS [5] gives average figures of 33 and 21 years for the lengths of time necessary for clinical emergence of IgG and IgA myeloma respectively from a single mutant cell These intervals are calculated from paraprotein doubling times and inferred tumour growth rates and represent the means of very wide ranges Hence, in our cases, the mutant cell could have appeared at about the same time or equally, widely spaced in time

In conclusion, it must be stated that it is likely that cases occurring in relatives are due simply to chance

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Acta Haemat 49 182 189 (1973)

Myelofibrosis, Osteolytic Bone Lesions and Hypercalcemia in Chronic Myeloid Leukemia

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Abstract A patient with chronic myeloid leukemia of 5 years duration developed myelofibrosis shortly before death Severe destructive bone lesions were found preterminally in the femur, ribs pelvis and skull Hypercalcemia of 14 2 mg% was present as well Although the serum calcium level was reduced by treatment to 11 5 mg% the patient died in renal failure and pulmonary infection shortly thereafter Hypercalcemia with its clinical implications should be suspected in the presence of myelofibrosis and bone pain complicating chronic myeloid leukemia

Key Words
Bone lesions in leukemia
Busulfan
Hypercalcemia
Myeloid leukemia
Myelofibrosis

Hypercalcemia, sometimes associated with destructive bone lesions, has been reported to occur in various forms of chronic leukemia [1, 16 30] Osteolytic lesions have also been encountered in a few cases of myelofibrosis developing as a late complication of chronic myeloid leukemia, unfortunately, data on serum calcium levels are not available in these cases [6, 14, 21] The present report describes a patient with chronic myeloid leukemia complicated preterminally by hypercalcemia, osteolytic lesions and myelofibrosis The clinical course and the management are discussed

Case Report

A 40-year old nun of Arab origin was first examined in January 1966, following the incidental finding of leukocytosis Physical examination revealed mild pallor There was no lymphadenopathy and the liver and the spleen were not palpable Hemoglobin 11 2 g% white blood cells 41 000 mm³ platelets 800 000 mm³ Differential count 2% myeloblasts 14% myelocytes 8% metamyelocytes 7% band

form, 48% polymorphonuclear cells, 3% eosinophils, 8% basophils, 9% lymphocytes and 1% normoblasts. A bone marrow aspirate revealed proliferation of the myeloid and megakaryocyte series and a slight depression of the erythroid series. The leukocytes did not stain for alkaline phosphatase. Cytogenetic analysis of bone marrow cells disclosed the presence of Philadelphia (Ph^1) chromosome. Serum calcium and phosphorus were 10 and 4.3 mg%, respectively. X-ray examinations of the chest and the long bones showed no abnormalities. The diagnosis of chronic myelogenous leukemia was established and therapy with 6 mg of busulfan daily was initiated. During the third month of therapy the platelet count decreased to 450,000 and the leukocytes to 5,850/mm^3. The patient continued to receive 2 mg of busulfan daily irregularly until 1968.

Physical examination in March 1968 revealed for the first time a palpable spleen. The hemoglobin was 10.8 g%, the leukocyte count 6,500/mm^3, the differential count was normal and the platelet count was 1.2 million/mm^3. Busulfan therapy was reinstituted and the platelets decreased gradually to 600,000/mm^3. Between 1968 and 1971 the patient suffered from repeated respiratory and urinary tract infections and allergic reactions to furadantin and ampicillin. In February 1971 she was hospitalized because of fever, arthralgia, aphthous stomatitis and erythema nodosum. Hemoglobin 11.6 g%, hematocrit 36%, platelets 240,000/mm^3 and leukocytes 5,900/mm^3. Differential count: 9% myeloblasts, 55% polymorphonuclear cells, 7% band form, 2% eosinophils and 30% lymphocytes. The bone marrow was hypercellular with a marked shift to the left in the myeloid series, suggesting exacerbation of leukemia. The sedimentation rate was 100 after the first hour (Westergren). Serum vitamin B_{12} was 5000 pg/ml and unsaturated vitamin B_{12} binding capacity 1450 pg/ml. Blood urea nitrogen (BUN) 28 mg%, uric acid 3.7 mg%, calcium 8.8 mg%, phosphorus 3.5 mg%, alkaline phosphatase 87 units (normal <85). Therapy with 30 mg prednisone and 150 mg 6-mercaptopurine daily was instituted. After 2 weeks all skin lesions disappeared and the patient was discharged.

Her final admission was on the 15th of April 1971 because of severe dehydration and oral moniliasis. While there were no significant changes in the hemoglobin level and the platelet count, a leukopenia of 2,700/mm^3 was found and the differential count showed 10% myeloblasts, 4% myelocytes, 10% basophils, 8% eosinophils, 8% band form, 4?% polymorphonuclear cells, 8% lymphocytes and 2% monocytes. Repeated attempts to obtain bone marrow by sternal punctures failed and a trephine biopsy of the iliac bone showed a group of myeloblasts and many myelocytes (Fig. 1). Urinalysis, serum proteins, bilirubin and electrolytes were within normal limits. The BUN was 63 mg%. The patient received intravenous fluids containing 150 mg hydrocortisone daily. A few days after admission she became weak and confused and complained of severe bone pain. Additional laboratory examinations showed a serum creatinine of 2.1 mg% and a creatinine clearance of 19 ml/min. Uric acid 7.7 mg%, calcium 14.2 mg%, phosphorus 3.9 mg% and alkaline phosphatase 95 units. Destructive bone lesions were demonstrated in the femur, ribs, pelvis and skull (Fig. 2). Treatment with a low calcium diet, infusion of saline and larger doses of hydrocortisone (300 mg/day) relieved the bone pain and improved the patient's general condition. Within the following 3 weeks the BUN dropped to 36 mg%, the blood creatinine to 1.7 mg%, the calcium to 11.5

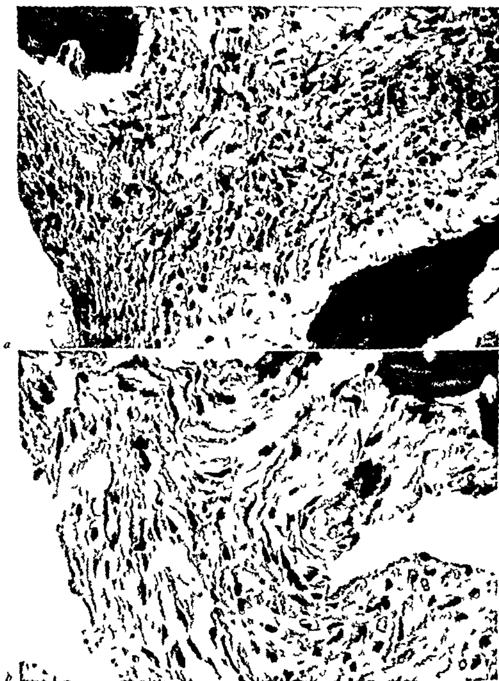


Fig. 1. a Survey of bone marrow showing hypocellularity and areas of fibrosis. HE × 340. *b* Detail of figure 1a showing fibrosis completely replacing hematopoietic tissue. HE × 580.



Fig. 2. X-ray film of skull showing multiple osteolytic lesions.

mg% and the phosphorus to 2.7 mg% (table I). The diuresis ranged between 1,500 and 2,500 ml. During this period the daily urinary excretion of calcium was in the range of [illegible] mg and that of phosphorus around [illegible] mg. Despite the decrease in serum calcium the creatinine clearance remained 19.6 ml/min and the urine osmolality 245 mOsm/kg H_2O. The patient continued to suffer from bone pain, dyspnea, tachycardia, cough and purulent sputum and expired on May [illegible], 1971. Permission for autopsy was not granted.

Discussion

Hypercalcemia is most commonly associated with malignant diseases. According to [illegible] [23], [illegible]% of patients with hypercalcemia had

Table I Serum biochemical values in the course of the disease

Date	Albumin g%	BUN, mg%	Uric acid mg%	Calcium, mg%	Phosphorus mg%	Alkaline phosphatase, units (n = 85)
15 1 66	3 2	21	5 5	10 0	4 3	72
15 2 71	3 0	28	3 7	8 8	3 5	93
20 4 71	3 3	60	7 2	14 2	3 9	95
25 4 71	2 6	46	6 2	13 1	3 6	137
10 5 71	2 3	36	5 2	12 1	2 7	145
18 5 71	2 4	26	5 5	11 5	2.1	135
20 5 71 death						

some kind of malignant disease while only 20% suffered from primary hyperparathyroidism Breast cancer accounts for over half of the cases, followed, in frequency, by carcinoma of the lung and the kidney [24] Leukemia is amongst the least frequent causes of hypercalcemia The first case was described by CLARK in 1936 [4] in a child who suffered from 'aleukemic' leukemia Since then more than 50 additional cases suffering from all forms of leukemia have been reported [16] Hypercalcemia occurs in 2 5–4 5% of cases with acute leukemia [19, 32] but is very rare in the chronic forms To the best of our knowledge, only 4 cases with chronic lymphatic [2, 7, 15, 33] and 5 with chronic myeloid leukemia [1, 16, 30] associated with hypercalcemia have been reported Osteolytic lesions in chronic myeloid leukemia have been reported by CLEMENTS and KALMAN [5] who collected a total of 7 cases from the literature Unfortunately, data on serum calcium levels were not reported but, at autopsy, deposition of calcium was found in many parenchymatous tissues

The cause of hypercalcemia in malignant diseases in general and in leukemia in particular is not clear In acute leukemia rapid destruction of bone may result in release of excessive amounts of calcium into the circulation The frequent finding of osteolytic lesions supports this assumption [32] However no meaningful correlation was found between the X-ray findings the high serum calcium levels and the bone changes at autopsy [2, 25] In one case the finding of extensive leukemic infiltration of the parathyroid gland was thought to have induced excessive

release of parathyroid hormone [28]. This finding was not confirmed in other cases where the parathyroid glands were normal in spite of the presence of hypercalcemia [1, 2, 16, 29]. The possible production and secretion of a parathyroid like hormone from leukemia cells, similar to that in carcinoma of the bronchus, malignant lymphoma, etc., has been suggested [26]. Another hypothesis assumes that leukemic cells may synthesize a substance similar to the osteolytic sterol in patients with breast cancer which causes bone destruction [13].

Clinical signs associated with hypercalcemia are related to the kidneys, the central nervous, gastrointestinal and cardiovascular systems. Early recognition of these symptoms is important because of the mortality which is high and increases considerably if treatment is delayed [22]. The prognosis of leukemia associated with hypercalcemia is poor. The median survival in acute leukemia was found to be no more than 3 months, while in the 4 cases of chronic myeloid leukemia it was less than 3 weeks [11].

In the present case, hypercalcemia was discovered shortly before death and more than 5 years after the diagnosis of chronic myeloid leukemia had been established. At this stage, a change in the type of the malignant process, from chronic myeloid leukemia to myelofibrosis, had taken place. A relationship between chemotherapy, particularly busulfan, and the development of myelofibrosis has been suggested [14]. The massive destruction of bones, which in the present case reminded the 'punched out' lesions in multiple myeloma, suggests that infiltration with either leukemic cells or fibroblasts resulted in severe osteolysis and release of calcium into the circulation.

Five of 14 reported patients who developed myelofibrosis in the course of chronic myeloid leukemia had radiographic bone lesions, either osteolytic, osteoblastic or predominantly sclerotic [14]. However, no data on serum calcium levels in these patients were given.

When hypercalcemia occurs in association with leukemia, the treatment should be aimed primarily at the leukemic process [7, 19, 22]. Additional therapeutic measurements such as infusions of saline [3], phosphate [12, 17], sulfate [18], EDTA [9], and furosemide [31] can be used in an attempt to increase urinary excretion of calcium. Mithramycin [10, 27] and corticosteroids [8, 23] have been also used. In the present case, although the serum calcium level decreased from 14.2 to 11.5 mg%, there was already irreversible damage to the kidneys, possibly caused by deposition of calcium in the tubes.

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H. VINAZZER: Gerinnungsstörungen in der Praxis. Fischer, Stuttgart 1972. 185 pp., 22 fig.

Nach den Worten des Verfassers soll das vorliegende Buch «das gesamte Gebiet der Blutgerinnung abhandeln und die derzeitigen Kenntnisse der Physiologie, Pathologie, Klinik, Diagnostik und Therapie in kurzgefasster Form darstellen». Das Buch soll also in erster Linie den praktizierenden Internisten und Hämatologen ansprechen. Die Hauptkapitel enthalten in mehr oder weniger ausführlicher Darstellung Zusammenfassungen über die Physiologie der Hämostase, den gezielten Untersuchungsgang bei hämorrhagischen Diathesen, die Methodik der Hämostaseabklärung und schliesslich eine Systematik der Blutungsübel und thromboembolischen Erkrankungen und deren Behandlung. Entsprechend dem Lehrziel des Buches beschränkt sich das Literaturverzeichnis auf die Angabe von 31 Lehrbüchern und Monographien sowie von 129 relevanten Originalarbeiten.

Die Darstellung ist übersichtlich und doch nicht zu knapp. Die Erwähnung nicht gesicherter Hypothesen ohne Literaturangaben im theoretischen Teil muss als Konzession im Rahmen der Gesamtgestaltung toleriert werden. Etwas arbiträr erscheint allerdings die Empfehlung verschiedener Gerinnungstests für die Allgemeinpraxis (P), die Fachpraxis (FP) und das spezialisierte Gerinnungslabor (GL). So gehört nach diesem Buch die Rekalzifierungszeit in die Kategorie P, die Thromboplastinzeit nach Quick jedoch zur Gruppe FP!

Noch steht eine international akzeptable Standardisierung der Gerinnungsuntersuchungen aus. Auch der Verfasser dieses Buches gibt gewissen Tests aufgrund persönlicher Erfahrungen den Vorzug, was ihm beim jetzigen «Stand des Unwissens» auf diesem Gebiet sicher nicht angekreidet werden kann. Bedauerlich ist allerdings die Darstellung der Fibrinstabilisierung in Abbildung 4 (Bildung von Disulfidbrücken anstelle der heute gesicherten Transamidierung). Auch an anderen Stellen finden sich unbedeutendere Unstimmigkeiten im Text (fehlende Beschriftung in Abbildung 22, schwankende Angaben über Normalwerte). Gesamthaft darf das Buch dem kritischen Leser als anregende Lektüre empfohlen werden. E. A. BECK, *Bern*

M. LEVITAN and A. MONTAGU: Textbook of Human Genetics. Oxford University Press, New York 1971. 931 pp., 279 fig., $ 15.00.

This book outlines the basic principles of genetics and provides an excellent introduction to recent developments in this area. It includes information on most of the single gene-determined disorders and their mechanism of inheritance. Some chapters (15 and 16) are of particular interest to hematologists, although they are not to be regarded as reference material. The book would benefit from more detailed accounts on somatic cell hybridization and the use of up-to-date techniques of chromosome analysis. J. MITRA, *New York*

International Committee for Standardization in Hematology (ICSH)
International Federation of Clinical Chemistry (IFCC)
World Association of (Anatomic and Clinical) Pathology Societies (WAPS)

Recommendation for Use of SI in Clinical Laboratory Measurements

Representatives of ICSH, IFCC and WAPS met in combined session in Munich on 14th September 1972, on the occasion of the VIIIth World Congress of Anatomic and Clinical Pathology. They agreed to recommend to the medical practitioners and all others concerned with health services throughout the world the following principles with regard to units of measurement for medical laboratory results.

1. The *International System of Units* (SI) is accepted in its broad application.

2. In accordance with chemical usage the preferred unit of volume is *litre*, symbol l and l[3].

3. For multiples and submultiples of units, including derived units, only one prefix should be used. For preference this should be confined to the numerator; an exception is made in the case of the kilogram. Thus units of concentration should use the litre as the denominator[1].

4. For quantities concerning a component with sufficiently well known chemical structure, molecular kinds of quantities, based on amount of substance (using the unit mole), are recommended[1].

5. In principle, a report of a determined quantity should always include information on (1) system, (2) component, (3) kind of quantity, (4) numerical value and (5) unit. Thus, as an example, plasma iron can be reported as: Plasma-Iron (II + III), substance concentration = 23 µmol/l (abbreviated: P-Iron (II + III), subst. c. = 23 µmol/l)[1].

6. The 3 organizations named above agree to consult in the future before advising on implementation of recommendations on quantities and units. For this purpose, a Liaison Committee on Quantities and Units will be established consisting of one representative of each of the 3 bodies.

7. Further information regarding specific recommendations can be obtained from the representative of the appropriate organization, as follows:

ICSH: Dr. S. M. Lewis, Royal Postgraduate Medical School, Hammersmith Hospital, London W 12 (England)

[1] Because of uncertainties [illegible] the elementary entity of hemoglobin to be used in [illegible], ICSH recommends that for the time being hemoglobin concentration in blood should be expressed as mass concentration, i.e. hemoglobin g/dl. It is, however, permitted to use substance concentration (e.g. µmol/l) [illegible] the elementary entity [illegible] should be specified.

[2] [illegible] should be given for [illegible] use. The [illegible] should be [illegible] and [illegible]

IFCC Dr R Dybkaer Department of Clinical Chemistry, Geriatric Unit De Gamles By, Nørre Alle 41, *DK-2200 Copenhagen N* (Denmark),

WAPS Dr J R Schenken Nebraska Methodist Hospital 3612 Cuming Street *Omaha NB 68131* (USA)

Munich September 14 1972

For ICSH O W van Assendelft, R J Eilers, A von Klein Wisenberg S M Lewis J Spaander

For IFCC H Büttner R Dybkaer A H Holtz

For WAPS G Astaldi K G von Boroviczény B E Copeland G J Corley, P I A Hendry C E D Taylor F D White

International Society of Haematology

The Second Meeting of the European and African Division of the International Society of Haematology will take place in Prague (Czechoslovakia) from August 27 to 29, 1973

Programme etiology, pathogenesis and treatment of leukaemia haemocoagulation and fibrinolysis immunology of leucocytes and transplantation problems, surface structures of lymphoid cells and their function A symposium on human red cell blood groups on the occasion of 100th anniversary of Dr Janský will be held during the congress

For information apply at the Czechoslovak Medical Society J E Purkyně Sokolská 31 *Prague 2* (Czechoslovakia)

Pappenheim-Preis 1972

Anlasslich der 16 Jahrestagung der Deutschen Gesellschaft fur Hamatologie wurde der Pappenheim Preis 1972 der von den Nordmark Werken Hamburg mit einem Geldbetrag dotiert wird an Herrn Dr Kay Brune Basel fur seine Arbeit «Hühnergranulozyten ein Modell fur die antimikrobielle Funktion peroxidasefreier menschlicher Granulozyten» verliehen

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Enzymatic Abnormalities in Megakaryocytes[1]

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Abstract. Cytochemical studies of various enzymes were carried out on megakaryocytes obtained from patients with disorders of megakaryocytes. In patients with idiopathic thrombocytopenic purpura megakaryocytes have uniformly low levels of the enzymes studied, as estimated by formazan deposition. In contrast, megakaryocytes obtained from patients with agnogenic myeloid metaplasia and especially essential thrombocythemia showed elevated enzymatic activities, particularly those of glycolytic enzymes such as lactic dehydrogenase. These findings presumably reflect the neoplastic nature of these myeloproliferative megakaryocytes.

Key Words
Agnogenic myeloid metaplasia
Cytochemistry
Megakaryocyte enzymes
Thrombocythemia
Thrombocytopenic purpura

This report describes cytochemically determined enzymatic abnormalities of megakaryocytes obtained from patients with idiopathic thrombocytopenic purpura (ITP), agnogenic myeloid metaplasia, and essential thrombocythemia.

Materials and Methods

Bone marrow was obtained by needle aspiration from the sternum or iliac crest of patients with disorders of megakaryocytes, all of which were characterized by increased numbers of megakaryocytes in the bone marrow. They included [illegible] patients with ITP, 4 patients with agnogenic myeloid metaplasia, and 4 patients

[1] Supported by American Cancer Society Institutional Research Grant IN [illegible] K, The University of Michigan Cancer Research Institute, and by a Faculty Research Grant, The Horace Rackham School of Graduate Studies, The University of Michigan, Ann Arbor, Mich.



Fig 1 Normal megakaryocyte Wright s stain × 1 200
Fig 2 Normal megakaryocyte LDH stain illustrating numerous formazan granules staining black indicative of enzymatic activity × 1 200

with essential thrombocythemia Bone marrow specimens obtained from 2 presumably normal patients undergoing evaluation for osteoporosis unrelated to obvious dysproteinemia were used as controls

Films of marrow flecks were made between methanol-cleaned glass coverslips and air dried Some of the flecks were stained with Wright s stain for light microscopic examination Others were fixed for 30 sec in a mixture of ethanol and dry ice at 70 °C and air dried These films were stained for lactic dehydrogenase (LDH) succinic dehydrogenase (SDH) malic dehydrogenase (MDH) α glycerophosphate dehydrogenase (mitochondrial NAD independent α GPD M) α glycerophosphate dehydrogenase (soluble NAD-dependent α GPD S) glucose-6-phosphate dehydrogenase (G6PD) and 6 phosphogluconate dehydrogenase (6PGD) according to the method of STUART and SKOWRON [1] and counterstained with a solution of 0 1% nuclear fast red (Schmid Co., Stuttgart) in 5% aqueous $Al_2(SO_4)_3$

Results

Light Microscopy

Normal bone marrow Megakaryocytes showed multiple lobules in their nuclei and blue to purple abundant cytoplasm containing numerous azurophilic and pink granules (fig 1)

Idiopathic thrombocytopenic purpura Most of the megakaryocytes were small resembling megakaryoblasts of normal marrows (fig 3)



Fig. 3. Megakaryocyte. ITP, showing large nucleus in relation to cytoplasm which contains numerous vacuoles and appears hypogranular. × 1,200.

Fig. 4. Megakaryocyte. ITP, demonstrating a paucity of formazan granules in the LDH stain. In addition these granules are in the perinuclear area. × 1,200.

Fig. 5. Megakaryocyte. Agnogenic myeloid metaplasia, demonstrating reticular character of nuclear chromatin. × 1,200.

Fig. 6. Megakaryocyte. Agnogenic myeloid metaplasia. LDH stain. The formazan granules are larger and more numerous than in the normal megakaryocyte. × 1,200.

Their cytoplasm was basophilic and demonstrated fewer granules than normal. Some megakaryocytes, however, did appear normal. In the majority of the megakaryocytes the nuclei had few or no lobules. Little evidence of platelet formation at the periphery of the megakaryocytes was seen. Cytoplasmic vacuoles were frequent. Some of the megakaryocytes appeared lymphoid, with scant clear basophilic cytoplasm and a nucleus containing small block-like and linear aggregates of chromatin.

Agnogenic myeloid metaplasia. Megakaryocytes were numerous. They were relatively uniform in size and occurred in aggregates of [illegible]



Fig 7 Megakaryocyte essential thrombocythemia with abundant cytoplasm containing innumerable granules Multilobulation of the nucleus is also a prominent feature × 1 000

Fig 8 Megakaryocyte essential thrombocythemia LDH stain The formazan granules are innumerable, imparting a dense black speckled appearance to the cytoplasm × 1,000

Their nuclei had thick, widely separated strands of chromatin in a network arrangement resembling that seen in a reticulum cell or hemohistiocyte (fig 5) These 'reticuloid' megakaryocytes usually had one or no nuclear lobules Their cytoplasm stained light azure with abundant pink and azure-staining nonspecific granules In some of the megakaryocytes there appeared to be fibrillar structures in the cytoplasm which imparted a 'wavy' appearance to it

Essential thrombocythemia Megakaryocytes from these patients were numerous and were exceptionally large in size as seen in figure 7 The nuclei had many lobules, often as many as 15 or 20 The cytoplasm was richly granular and voluminous Frequently, aggregates of platelets appeared at the cell periphery

Cytochemical Studies

Table I summarizes the results of cytochemical studies performed on these megakaryocytes As estimated by formazan deposition, LDH activity was 'intermediate' in amount in normal megakaryocytes (fig 2) LDH activity was lowest in megakaryocytes obtained from patients with ITP, and the few formazan granules present appeared to be in a perinu-

Table I. Cytochemical studies of various enzymes in megakaryocytes

	LDH	SDH	MDH	G6PD	6PGD	α-GPD-M	α-GPD-S
Normal	2+	2+	0	2+	2+	2+	2+
ITP	0-1+	1+	0	1+	1+	1+	1+
Agnogenic myeloid metaplasia	3+	1+	0	1+	3+	3+	3+
Essential thrombocythemia	4+	1+	0	1+	4+	4+	3+

0 = No formazan granules seen. 1+ = Less than 10 granules seen. 2+ = 20–100 granules seen. 3+ = 100–500 granules seen. 4+ = Greater than 500 granules seen.

clear distribution (fig. 4). LDH activity was considerably higher than normal in megakaryocytes obtained from patients with agnogenic myeloid metaplasia (fig. 6). In patients with essential thrombocythemia LDH activity was highest (fig. 8). The megakaryocytes contained innumerable black formazan granules, as seen in figure 8. Similar intense LDH activity was observed in platelet aggregates found on the marrow films of these patients. α-GPD-S and α-GPD-M activity was highest in essential thrombocythemia megakaryocytes. 6PGD activity was also increased in these cells compared to normal and to ITP megakaryocytes. G6PD and SDH activities were very low in all types of megakaryocytes studied. MDH activity could not be demonstrated in any of the megakaryocytes.

Discussion

FRANK [2] first described the appearance of megakaryocytes in ITP. He noted that many of them had a decreased number of cytoplasmic granules and appeared immature. [illegible] [3] also noted a similar decrease in megakaryocytic granules in ITP. DAMESHEK and MILLER [4], [illegible] *et al.* [5, 6], DIGGS and HEWLETT [7] and PISCIOTTA *et al.* [8] have described megakaryocytes in ITP as being hypogranular and immature. The present studies of ITP megakaryocytes support these observations and in addition point to enzymatic differences which distinguish the ITP megakaryocyte from megakaryocytes in the other disorders studied. The studies just described suggest that the ITP megakaryocytes

are enzymatically 'young' when compared to normal megakaryocytes Although the number of formazan granules indicating enzymatic activity in the ITP megakaryocytes appeared to be fewer in number than the normal young megakaryocyte, it is difficult to say with certainty that the ITP megakaryocyte differed substantially from the normal megakaryoblast, at least with respect to the enzymes studied cytochemically

Although several reviews of essential thrombocythemia as a clinical entity have appeared [9–11] there has been little information regarding the characteristic features of the megakaryocytes seen in this disorder The cytochemical studies described indicate that the unusually large megakaryocytes of essential thrombocythemia have marked increase in LDH, 6PGD, and GPD M when compared to the megakaryocytes in the other conditions studied The increase in LDH is especially striking and can be seen in both the megakaryocyte cytoplasm and in the platelet aggregates in these patients Agnogenic myeloid metaplasia megakaryocytes showed a similar although less marked increase in activity of these enzymes

The increase in 6PGD activity suggests an increased activity of the pentose phosphate shunt pathway of energy metabolism in these myeloproliferative megakaryocytes The increased enzymatic activities may also relate to the increased platelet proliferative capacity of these cells In particular the increase in enzymatic activity of enzymes in the glycolytic pathway has been observed in neoplastic cells by others [12 13] and has been considered to be a hallmark of neoplasia from the enzymatic standpoint The increase in the activity of glycolytic enzymes (LDH and α GPD M) in both the agnogenic myeloid metaplasia and essential thrombocythemia megakaryocytes further supports the view that these megakaryocytes are neoplastic

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Die Aktivität der Methionin-Synthetase (5-Methyl-5,6,7,8-tetrahydrofolsäure: Homocystein Methyltransferase) als *Proliferationsparameter in wachsenden Zellen*[1]

H. SAUER, K. WILMS, W. WILMANNS und L. JAENICKE

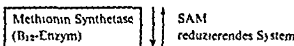
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Medizinische Universitätsklinik II, Tübingen

Abstract In cultured human lymphoblasts methionine synthase (MS) activity is enhanced during the logarithmic phase of population growth. MS is a 'log-phase enzyme'. Little activity is found in normal leukocytes and in cells of patients with chronic myelocytic leukemia. Acute myelocytic and lymphocytic leukemias show markedly enhanced values. Compared to thymidine kinase and DNA polymerase, the very stable MS gives the same possibility to control the treatment of leukemic patients.

Key Words
Folic acid
Leukemia
Leukocyte proliferation
Methionine synthetase

Die Methionin-Synthetase (EC noch nicht festgelegt), ein durch S-Adenosylmethionin (SAM) aktivierbares Cobalamin-Enzym [4, 7, 10, 12, 17, 18], katalysiert folgende Reaktion

N^5-Methyl 5,6,7,8 tetrahydropteroylglutaminsäure ($CH_3 FH_4$) + Homocystein



5,6,7,8-Tetrahydropteroylglutaminsäure (FH_4) + Methionin.

Dabei entsteht als Produkt neben Methionin auch Tetrahydrofolsäure. Diese wird durch die Methyltransferase aus der als 5-Methyl-Tetrahydrofolsäure vorliegenden Speicherform der Folsäure [2, 15] freigesetzt. Sie ist der im Stoffwechsel der Einkohlenstoff(C_1)-Einheiten und bei der Bildung von Glycin wirkende Kofaktor. Die für die *De-novo-*

[1] Mit Unterstützung der Deutschen Forschungsgemeinschaft

Synthese von Purinen und Thymidylat nötigen Tetrahydrofolsäure. Derivate Formyl-, Methenyl- und Methylen-Tetrahydrofolsäure werden aus ihr durch weitere enzymatische Umwandlungen gebildet. Die Zusammenhänge zwischen Folsäurestoffwechsel und Nukleotidsynthese sind in Abbildung 5 schematisch dargestellt.

Während der logarithmischen Wachstumsphase von Bakterien und Zellkulturen kann eine deutliche Zunahme der Aktivität von Enzymen festgestellt werden, die mit der Nukleinsäuresynthese in Zusammenhang stehen [6, 16]. Einige dieser sogenannten Log-Phasen-Enzyme sind: Dihydrofolsäure-Reduktase (EC 1.5.1.3), 10-Formyl-Tetrahydrofolsäure-Synthetase (EC 6.3.4.3), 5,10-Methylen-Tetrahydrofolsäure-Dehydrogenase (EC 1.5.1.5), Serin-Hydroxymethyl-Transferase (EC 2.1.2.1), Desoxyribonukleinsäure-Polymerase (EC 2.7.7.7), Thymidin-Kinase (EC 2.7.1.21). Es ist zu erwarten, dass in einer schnell proliferierenden Zellpopulation mit hoher DNS-Syntheserate auch die Methionin-Synthetase in erhöhter Aktivität vorliegt, um ausreichend hohe Konzentrationen der C_1-aktivierenden und -transportierenden Koenzyme zu gewährleisten. Wir fanden hohe Aktivitäten der Methionin-Synthetase in schnell wachsenden Geweben [3, 13] und in Zellen von Patienten mit akuten Leukosen eine gesteigerte Aktivität gegenüber normalen Populationen weisser Blutzellen [13]. Alle genannten Enzyme werden auch im normalen Stoffwechsel der Zellen benötigt. Ein Nachweis erhöhter Aktivitäten kann also nicht spezifisch für einen malignen Prozess sein, sondern es sind nur Aussagen über die jeweilige momentane Wachstumstendenz normaler oder pathologischer Zellen möglich.

Durch Untersuchungen an Zellkulturen und durch Verlaufsbeobachtungen an Patienten mit akuten Leukämien wird in der vorliegenden Arbeit die Frage untersucht, ob die Methionin-Synthetase tatsächlich zu den Log-Phasen-Enzymen gerechnet werden kann. Als Bezugssysteme dienen dabei die DNS-Polymerase und die Thymidin-Kinase. Diese beiden Log-Phasen-Enzyme wurden gewählt, da sie als gute Parameter für die Beurteilung der Proliferationstendenz einer Leukozytenpopulation bekannt sind [6, 19, 24].

Methoden und Material

Weisse B[illegible] Zellk[illegible] werden nach Wilmanns [illegible] Patienten mit akuter [illegible] Leukämie [illegible]

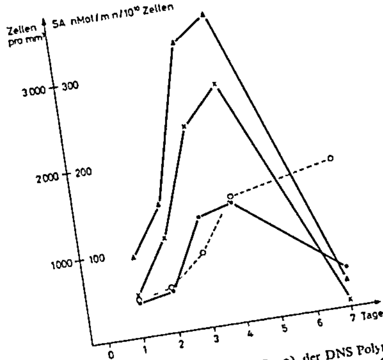


Abb 1 Aktivität der Methionin Synthetase (MS = ●), der DNS Polymerase (▲) und der Thymidin Kinase (TK = ×) während einer Wachstumsperiode einer SK-L1-Lymphoblastenkultur SA = Spezifische Aktivitäten der Enzyme, ○ = Zellzahl

loischer Leukämie (RPMI 6410-Linie) [11] und Burkitt Lymphom (Burkitt Linie wurden in RPMI 1640-Medium, erganzt mit 15% fetalem Kälberserum (beides vo Microbiological Associates, Bethesda), gezuchtet Die Bestimmung des Anteils tot Zellen in den Kulturen erfolgte durch Anfärbung mit 1% Trypanblau in RPM 1640 Medium

Zur Stimulierung von Lymphozyten mit Phythamagglutinin wurde nach Wi und WILMANNS [25] verfahren

Die enzymatischen Aktivitaten in den zellfreien Extrakten wurden nach fol den Methoden ermittelt DNS-Polymerase nach WILMS und JAENICKE [24] Th din Kinase nach WILMANNS [19], Methionin Synthetase nach SAUER und JAE [13]

Ergebnisse

Zellkulturen Wahrend des Wachstums zeigten alle drei Kultur Beginn der logarithmischen Wachstumsphase eine starke Aktivi nahme der Methionin-Synthetase, ahnlich wie es bei den zum Ve

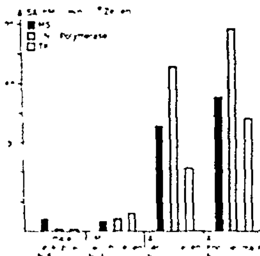


Abb. 2 Proliferationsstoffwechsel Enzyme in normalen und pathologischen Zellen des Blutes und des Knochenmarkes. CML = Chronische myeloische Leukämie. AL = akute Leukosen. N = Anzahl der untersuchten Fälle

gemessenen Aktivitäten von DNS Polymerase und Thymidin Kinase der Fall ist. Abbildung 1 zeigt den Verlauf während einer Wachstumsperiode der SK L, Kultur. Hier erreicht die Methionin Synthetase das 3fache, die DNS Polymerase das 4fache und die Thymidin Kinase das 5,5fache ihres Anfangswertes. Die Änderung aller Enzymaktivitäten ist gleichsinnig, wenn schon bei der Methionin Synthetase am geringsten. Die Methionin Synthetase ist zu den Log Phasen Enzymen zu rechnen. Nach Abbildung 1 darf somit auch die Methionin Synthetase Aktivität als Proliferationsparameter angesehen werden. Die Abnahme der Enzymaktivitäten mit dem Wachstumsstillstand beruht dabei nicht auf einem Absterben der Zellen, denn während der ganzen Untersuchungsperiode wurden konstant 90% vitale Zellen in den Kulturen festgestellt. Dagegen werden die Zellteilungszeiten länger oder kommen ganz zum Stillstand. Ursache der verminderten enzymatischen Aktivitäten können verschiedene Mechanismen sein. Einmal kann ein am Enzym selbst angreifende kompetitive Hemmung oder eine allosterische Feedback Hemmung durch sich anhäufende Metabolite (z. B. Nukleosidphosphate) von Bedeutung sein. Andererseits können die gleichen Substanzen auch Effekto-

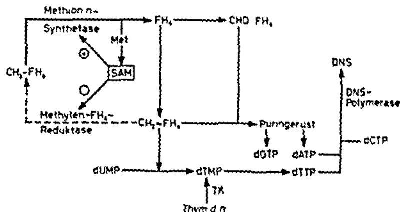


Abb 5 S-Adenosylmethionin (SAM) als Regulator in der Bildung von «aktiven» Einkohlenstoff-Einheiten FH_4 = Tetrahydrofolsäure, CH_2 = FH_4 = Methylentetrahydrofolsäure, CH_3 FH_4 = Methyltetrahydrofolsäure, CHO FH_4 = Formyltetrahydrofolsäure, TK = Thymidin Kinase, – = Hemmung + = Aktivierung

Dies wird besonders deutlich bei Verlaufsstudien an entsprechenden Patienten In dem in Abbildung 3 wiedergegebenen Fall steigt parallel zum Einsetzen eines neuen Schubes mit Ausschwemmung vieler unreifer Blasten in die Peripherie die Aktivität der Methionin-Synthetase genau so wie die der Thymidin-Kinase Unter einer effektiven Therapie mit Zytosinarabinosid in Kombination mit 6-Thioguanin fallen die Enzymaktivitäten wieder ab

Darüber hinaus konnten wir in einem weiteren Fall (Abb 4) zeigen, dass eine im Vergleich zu Vorwerten stark gesteigerte enzymatische Aktivität im Knochenmark ein bis zwei Tage später zu einem massiven leukämischen Schub in der Peripherie führte Es scheint also möglich, durch häufige Kontrolle der Enzymaktivitäten der Leukozyten des peripheren Blutes und des Knochenmarkes einen akuten Schub bei Leukämien vorauszusehen und therapeutische Konsequenzen zu ziehen

Diskussion

Unsere Untersuchungen bestätigen die Annahme, dass die Methionin-Synthetase eine wichtige Funktion während der Zellteilung hat Durch die Bereitstellung der «essentiellen» Aminosäure Methionin dient sie als Starter der Protein- und Enzymsynthese, durch die Freisetzung des Ko-

faktors Tetrahydrofolsäure bringt sie den C_1-Stoffwechsel in Gang. Dies ist vermutlich die wichtigere Aufgabe.

Wegen der zentralen Stellung der Methionin-Synthetase im Zellstoffwechsel hat auch ihre Regulation eine besondere Bedeutung. Über das entstehende Methionin und den mit Hilfe von ATP daraus gebildeten Kofaktor S-Adenosylmethionin kann der Stoffwechsel der C_1-Einheiten und so die *De novo*-Synthese der Nukleotide gesteuert werden. Die Aktivierung der Methionin-Synthetase und die gleichzeitige allosterische Hemmung der Methylen-Tetrahydrofolsäure-Reduktase (EC 1.1.1.68) [2] führen dazu, dass die Menge der Methyl-Tetrahydrofolsäure, die als Speicher- oder Ventilform der Tetrahydrofolsäure betrachtet werden kann, abnimmt. Aus Abbildung 5 ist ersichtlich, wie es durch den katalytischen und allosterischen Effekt des S-Adenosylmethionins zur Anreicherung derjenigen Folsäureverbindungen kommt, die im Synthesestoffwechsel verwendet werden, nämlich neben der Tetrahydrofolsäure selbst ihre Formyl- und Formaldehyd-substituierten Derivate (CHO-FH_4 und CH_2=FH_4).

Der zweite an der Methionin-Synthetase-Reaktion beteiligte Kofaktor ist das Vitamin B_{12}. Die bei Vitamin-B_{12}-Mangel auftretenden Zellteilungs- und Reifungsstörungen können ebenfalls durch die Schlüsselstellung der Methionin-Synthetase in der Neusynthese von Nukleinsäuren erklärt werden. Das Vitamin B_{12} ist im aktiven Zentrum des Enzyms als Kofaktor komplex gebunden und wird dort durch S-Adenosylmethionin methyliert [7]. Erst dieser stabilisierte [7, 16] Methyl-B_{12}-Enzym-Komplex kann katalytisch wirksam sein. Bei einem B_{12}-Mangel wird sich also Methyl-Tetrahydrofolsäure anhäufen. Da dann keine Tetrahydrofolsäure zum C_1-Transport freigesetzt werden kann, hat diese sogenannte »Methyl-Folat-Falle« (methyl folate trap) eine verminderte Purin- und Thymidylatsynthese zur Folge. Die wegen Substratmangel unzureichende DNS-Synthese wird die bekannten Störungen der Zellproliferation [illegible]

Durch dessen katalytische Tätigkeit wird aus 5-Methyl-Tetrahydrofolsäure Tetrahydrofolsäure freigesetzt und der C_1-Stoffwechsel wieder aktiv. Über die *De-novo*-Synthese von Thymidin-Methyl-Gruppen und daraus neu gebildetes Thymidintriphosphat kommt es schliesslich wieder zur normalen Feedback-Hemmung der Thymidin-Kinase.

Die in Substratkonzentrationen benötigte Methyl-Tetrahydrofolsäure hat unter den von uns geprüften Bedingungen (bis zu 10^{-3} M Methyl-Tetrahydrofolsäure im Kulturmedium) keinen Einfluss auf die Aktivität der Methionin-Synthetase. Da die Zellen in Medium mit 15% fetalem Kälberserum, das sehr reich an Folsäureverbindungen ist, gehalten wurden, können wir derzeit keine Aussage über die Enzymaktivität bei Methyl-Folat-Mangel machen. BERTINO und CHELLO [1] berichteten, dass nach Abbau der Folate im Medium durch Carboxypeptidase G_1 das Log-Phasen-Enzym Dihydrofolsäure-Reduktase früher und stärker ansteigt als in den Kontrollen ohne Folat-Mangel. Daraus wird die Hypothese abgeleitet, dass der Folat-Koenzym-Spiegel die Aktivität einiger Folat-abhängiger Enzyme regelt.

Auch in zellfreien Systemen *in vitro* wird die Methionin-Synthetase-Aktivität durch hohe Konzentrationen von Methyl-Tetrahydrofolsäure, Tetrahydrofolsäure oder von Homocystein und Methionin nicht beeinflusst. Eine Feedback-Hemmung oder eine Aktivierung durch die direkt an der Reaktion beteiligten Moleküle findet also nicht statt.

Folsäureanaloge (Aminopterin, Amethopterin = Methotrexat®) und deren tetrahydrierte Derivate hemmen die Methionin-Synthetase nicht. Dadurch ist es möglich, die zytostatische und toxische Wirkung von Methotrexat durch Methyl-Tetrahydrofolsäure aufzuheben [14]. Über die intakte Methionin-Synthetase-Reaktion wird genügend Tetrahydrofolsäure freigesetzt, so dass die durch den Folsäure-Antagonisten bewirkte Blokkierung der Dihydrofolsäure-Reduktase umgangen werden kann.

Zusammenfassung

Kultivierte menschliche Lymphoblasten zeigen einen deutlichen Anstieg der Methionin-Synthetase(MS)-Aktivität während der logarithmischen Wachstumsphase. Die MS ist ein «Log-Phasen-Enzym». In normalen peripheren Leukozyten ist nur wenig MS-Aktivität vorhanden, ebenso in Zellen von Patienten mit chronischer myeloischer Leukämie. Akute myeloblastäre und lymphoblastäre Leukämien zeigen stark erhöhte Werte. Die MS ermöglicht die gleiche Aussage über die Proliferationstendenz einer Zellpopulation wie die DNS-Polymerase und die Thymidin-

Kinase und gibt so die Möglichkeit, die Therapie akuter Leukämien zu überwachen. Vorteile ergeben sich aus der grossen Stabilität des Enzyms.

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Acta Haemat. 49: 211-214 (1973)

δ-Aminolaevulinic Acid Synthetase Activity in Normal Human Liver Homogenate

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Abstract. In a series of 20 normal subjects, the δ-aminolaevulinic acid synthetase activity in liver homogenate has been assayed. The values ranged from 8.9 to 29.6 nmoles δ-aminolaevulinic acid/g liver/h; mean value 19.66 ± SD 6.64. The results are compared to those published by other authors using the same or other methods.

Key Words
δ-ALA synthetase
Haem synthesis
Human liver
Porphyrin metabolism

δ-Aminolaevulinic acid synthetase (ALAS) is the rate limiting enzyme for haem synthesis in the liver. As publications on the assay of the enzyme in the normal human liver have been rather scanty, and the results conflicting, it has been considered worth while to carry out further determinations.

Material and Methods

Twenty non-porphyric subjects (12 males and 8 females) aged 34-49, undergoing laparotomy for various conditions, namely gastrectomy, appendicectomy, etc., have been included in this series. There was no history of liver disease, alcoholism or other factors known to influence liver porphyrin metabolism. A sample (1-2 g) of the macroscopically normal liver was immediately removed and placed in [illegible] homogenisation buffer. The subsequent steps were as described by Marver et al. [4].

Results

The results are given in table I. Enzyme activity ranged from 8.9 to 29.6 nmoles ALA/g/h, mean value 19.66 ± SD 6.64.

Table I ALAS activity in normal human liver

Subjects	Sex	Age, years	ALA, nmoles/g liver/h
D S	F	63	21.5
C S	F	40	19.21
M S	F	37	28.2
K A	F	38	13.0
K N	F	35	20.0
J L	F	32	18.3
M G	F	35	14.0
M C	F	48	28.9
P J	M	35	26.6
D K	M	63	8.9
R M	M	69	12.0
A H	M	34	24.9
K T	M	62	27.6
T G	M	59	25.3
K D	M	48	22.1
C G	M	25	21.6
M O	M	29	13.4
T J	M	65	11.7
L G	M	47	10.6
T L	M	32	22.4
Mean value			19.66 ± S.D. 6.64

Discussion

Tschudy *et al.* [7] were the first to measure liver ALAS activity in 7 normal subjects. They incubated liver homogenate under conditions where ALA utilisation is almost completely inhibited and the production of ALA is optimal. Other workers [3, 6] measured ALAS activity in a few cases using isolated mitochondria and adding glycine and Krebs cycle intermediates according to the technique of Granick and Urata [2]. By this method incubation is delayed and owing to the short half-life of liver ALAS much of the enzyme activity is lost. Dowdle *et al.* [1] using a micromethod for the assay of ALAS in liver needle biopsy specimens found no activity in 2 normal controls. This could be ascribed to contamination of the zero-time samples with various non-specific Ehrlich aldehyde re-

Table II. ALAS activity in normal human liver reported in the literature

Authors	Number of subjects	Material and methods	Hepatic ALA synthetase activity
[illegible] *et al.* [7]	7	macromethod (in liver homogenate)	15-31 nmoles ALA g/h
[illegible] [3]	2	GRANICK and URATA's method (in isolated mitochondria) [2]	0-1.16 nmoles aminoacetone 0.1 mm³¹ 1.5 h
[illegible] *et al.* [6]	3	GRANICK and URATA's method (in isolated mitochondria) [2]	zero to trace nmoles ALA mm³/120 min
DOWDLE *et al.* [1]	2	macromethod (in liver homogenate)	0 nmoles ALA/3.1 mg total liver phosphorus/h
MCINTYRE *et al.* [5]	not quoted	macromethod (in liver homogenate)	<5 nmoles ALA/g/h

¹ mm³ = ml packed cell volume.

[illegible] chromogens not separated by their technique. MCINTYRE *et al.* [5] have also reported on normal range of hepatic ALAS activity in humans, without mentioning the number of cases studied. The results of these authors are summarized in table II.

In the present study the method of MARVER *et al.* [4] was chosen since (a) the pyrrole of ALA (2-methyl-3-acetyl-4-propionic acid pyrrole) is separated from aminoacetone pyrrole (2,4-dimethyl-3-acetylpyrrole), (b) the amount of ALA produced per unit liver is somewhat greater than that produced by other methods and so it can permit tracing of even very little enzyme activity, and (c) the values of ALAS activity found by this method are close to that required for synthesis of haem enzymes under normal conditions [4].

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4 MARVER, H S, TSCHUDY, D P, PERLROTH, H G., and COLLINS, A δ-aminolaevulinic acid synthetase I Studies in liver homogenates J biol Chem *241* 2803–2809 (1966)

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7 TSCHUDY, D P, PERLROTH, M G, MARVER H S, COLLINS, A, HUNTER, G, jr., and RECHCIGL, M, jr Acute intermittent porphyria the first overproduction disease localized to a specific enzyme Proc nat Acad Sci., Wash *53* 841–847 (1965)

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Acta Haemat. 49: 215–222 (1973)

Factors Influencing Splenic Pooling of Erythrocytes in the Myelo- and Lympho-Proliferative Syndromes

P. J. TOGHILL and SHEILA GREEN

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Abstract. The splenic pool size has been measured in 50 patients with splenomegaly due to myelo- and lympho-proliferative disorders. Splenic pool size was related primarily to spleen size and was not significantly influenced by the pathological disorder. Changes in spleen size due to treatment or progressive disease altered the pool size. The splenic pool could be discharged with adrenaline and noradrenaline.

Key Words
Erythrocyte mass
Lymphomas
Myeloproliferative disorders
Spleen pool
Splenomegaly

The enlarged spleen in man contains a pool of concentrated erythrocytes in the pulp which slowly exchanges with blood in the faster flowing direct arterio-venous channels [7]. In this splenic pool the red cells are subjected to the potentially hostile effects of the intrasplenic environment and undergo changes which render them more susceptible to haemolysis [9]. A number of factors may influence the size of the splenic pool. It is likely that the largest pools exist in patients with gross splenomegaly and in those with haemolytic disorders. In contrast some patients with lympho-proliferative disorders may have comparatively small pools relative to spleen size [8]. There is also evidence that the splenic pool is in a dynamic equilibrium with the arterio-venous blood stream and may be discharged in a similar way to the reservoir spleen in certain animals [10]. This paper describes some factors which appear to influence splenic pooling in the myelo- and lympho-proliferative syndromes in man.

Methods and Materials

Patients. 50 patients with varying degrees of splenomegaly were studied. In the group of 29 patients with myeloproliferative disorders, 6 were suffering from poly-

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Abstract. The splenic pool size has been measured in [illegible] patients with splenomegaly due to myelo- and lympho-proliferative disorders. Splenic pool size was related primarily to spleen size and was not significantly influenced by the pathological disorder. Changes in spleen size due to treatment or progressive disease altered the pool size. The splenic pool could be discharged with adrenaline and noradrenaline.

Key Words
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The enlarged spleen in man contains a pool of concentrated erythrocytes in the pulp which slowly exchanges with blood in the faster flowing direct arterio-venous channels [7]. In this splenic pool the red cells are subjected to the potentially hostile effects of the intra-splenic environment and undergo changes which render them more susceptible to haemolysis [9]. A number of factors may influence the size of the splenic pool. It is likely that the largest pools exist in patients with gross splenomegaly and in those with haemolytic disorders. In contrast some patients with lympho-proliferative disorders may have comparatively small pools relative to spleen size [6]. There is also evidence that the splenic pool is in a dynamic equilibrium with the arterio-venous blood stream and may be discharged in a similar way to the reservoir spleen in certain animals [10]. This paper describes some factors which appear to influence splenic pools in the myelo- and lympho-proliferative syndromes in man.

Methods and Materials

Patients. [illegible] patients with varying degrees of splenomegaly were studied. In the group of [illegible] patients with [illegible] disorders, [illegible] were suffering from [illegible]

cythaemia 7 had chronic myeloid leukaemia 15 had myeloid metaplasia and one patient had primary thrombocythaemia In the group with lympho-proliferative disorders 14 were suffering from chronic lymphatic leukaemia and 7 from lymphosarcoma Spleen size was recorded as the maximum distance of the lower edge downwards and medially from the left costal margin Patients in whom the spleen tip was palpable on deep inspiration were included but were shown as having spleens 0 cm below the left costal margin Serial recordings were carried out in 11 patients in whom there were changes in spleen size of at least 5 cm below the left costal margin either as a result of therapy or of natural progression of the disease

Measurement of red cell mass (RCM) and splenic red cell pool (SRCP) Erythrocytes were labelled with 50–100 μCi sodium chromate ($Na_2{}^{51}CrO_4$) using the method of JOSKE *et al* [5] After the intravenous injection of a known volume of autogenous labelled cells into a forearm vein venous samples were taken from the opposite arm at intervals from 3 to 45 min after the mid point of the initial injection In all patients there was complete equilibration of erythrocytes in the splenic pool by 45 min and the activity of this sample was used to calculate the total RCM The curve obtained from the activities of the serial samples of blood taken during the 3- to 45 min period represented the phase of slow mixing in the spleen and retrapolation of this curve to zero time gave a value which could be used to calculate the extra splenic red cell mass [11] The difference between this figure and the RCM was taken to represent the SRCP

Dynamic studies of splenic pool Measurements of surface activity over the enlarged spleen were carried out using a collimated scintillation counter connected to a continuously recording ratemeter After the injection of autogenous labelled cells continuous radioactivity changes over the approximate geometrical centre of the surface marking of the spleen were recorded with the counter vertical and the subject supine Drugs were given by constant intravenous infusion with regular blood pressure monitoring

Results

Splenic pool size in myelo- and lympho proliferative syndromes The relationships between splenic pool size and spleen size in the myelo- and lympho-proliferative syndromes are shown in figures 1 and 2 A splenic pool of more than 5% of the total red cell mass was detected in 21 of the 29 patients with myelo-proliferative syndromes and 17 of the 21 patients with lympho proliferative syndromes In the myelo proliferative syndromes there was a highly significant relationship between the spleen size and the splenic pool size expressed as a percentage of the red cell mass ($r = 0.55$, $p < 0.001$) In the individual disease groups the relationship was significant in chronic myeloid leukaemia ($r = 0.78$, $p < 0.02$) but not significant in the group of patients with polycythaemia ($r = 0.28$) or myeloid metaplasia ($r = 0.30$) In those patients with lympho proliferative syn-

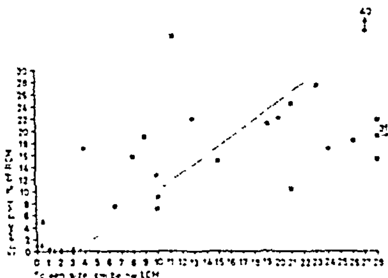
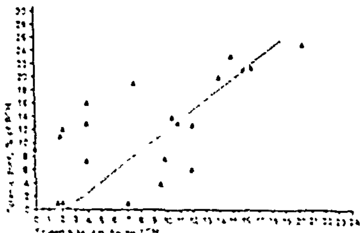


Fig. 1. The relationship between splenic pool size and spleen size in myeloproliferative disorders. ■ = myeloid metaplasia, ▲ = polycythaemia, ● = chronic myeloid leukaemia, ⧫ = thrombocythaemia.



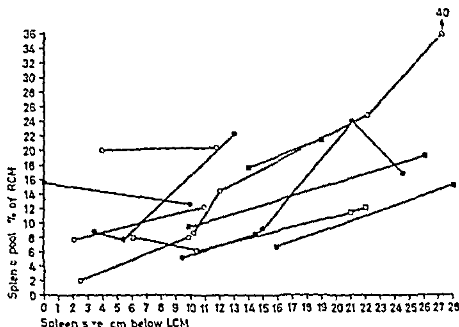


Fig 3 Serial changes in splenic pool size with changes in spleen size as a result of treatment or progressive disease ● = Chronic myeloid leukaemia, ■ = myeloid metaplasia ○ = chronic lymphatic leukaemia □ lymphosarcoma

dromes there was a highly significant relationship between spleen size and splenic pool size ($r = 0.67$, $p < 0.001$) The splenic pool size for corresponding spleen size was not significantly different in the myelo proliferative or the lympho-proliferative syndromes

Splenic pool size with serial changes in spleen size In 11 patients measurements of splenic pool size were performed at intervals over a period of 2 years during which significant changes in spleen size occurred as a result of therapy or of natural progression of the disease The results are shown in figure 3 In 8 patients the splenic pool showed an increase with increase in spleen size In 1 patient with chronic myeloid leukaemia the measured splenic pool rose from 12 5 to 15 5% of the red cell mass during treatment with busulphan in which the spleen shrank from 10 cm below the left costal margin to being impalpable In the remaining patient with lymphosarcoma no significant decrease in splenic pool occurred as the spleen shrank after radiotherapy

Mobilisation of the splenic pool by pharmacological agents In 3 patient volunteers the mobility of the splenic red cell pool was demonstrated during an infusion of noradrenaline at rates of 12–24 μg/min In each pa-

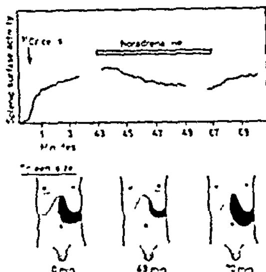


Fig. 4. Changes in splenic surface activity and spleen size during intravenous infusion of noradrenaline.

tient autogenous ⁵¹Cr labelled erythrocytes were injected intravenously and venous samples taken from the opposite arm at 2 min intervals. Simultaneous recordings of the surface activity over the spleen were carried out and in each case a slow rise in activity following the initial mixing phase indicated pooling of labelled erythrocytes in the spleen. During the infusion of noradrenaline there was a reduction in activity over the spleen corresponding approximately to the amount pooled in the spleen (fig. 4). Assuming no changes in plasma volume during the times of the experiments, it was calculated from the peripheral blood samples that 265, 130 and 200 ml erythrocytes were discharged from the spleens of these patients in whom the splenic pools had been calculated at 445, 95 and 215 ml (fig. 5). Similar results were obtained in 1 patient with adrenaline. From these experiments it seemed likely that the enlarged spleen in man could be made to contract under the influence of noradrenaline and adrenaline and that the blood extruded was probably derived from the splenic pool. Angiotensin given in dosages adequate to produce systolic hypertension of 200 mm did not produce splenic contraction, and β-blocking agents such as phenoxybenzamine and propranolol did alter the splenic pool size as measured by these methods.

Patient	Splenic pool, ml / Red cell mass ml	Volume of blood (ml) discharged from spleen
ML. CM leukaemia	445/2050 = 22%	265
TK. Myeloid metaplasia	95/1275 = 7%	130
PK. Myeloid metaplasia	215/1125 = 19%	200

Fig 5 Blood discharged from enlarged spleens during infusion of noradrenaline

Discussion

Although the concept of a splenic erythrocyte pool is now well accepted there is considerable variation in the method used for its measurement The technique used in this paper is that described earlier [11] in which the splenic pool is calculated from serial samples of peripheral blood taken during the slow mixing phase in the spleen following the rapid intravenous injection of autogenous ^{51}Cr-labelled erythrocytes Other authors have used essentially similar methods using labelled erythrocytes [2, 10] CHRISTENSEN [3] calculated the pool by surface splenic activity measurements with ^{51}Cr-labelled cells using appropriate correction factors for varying spleen size PETTIT *et al* [8] have used a scanning method with (^{11}C) carbon monoxide for the direct *in vivo* measurement which recorded the red cell content of the spleen rather than pool size In the consideration of factors controlling the splenic red cell pool it is, therefore, important to relate the results with the method used

Studies of splenic pool size in patients with specific diseases such as tropical splenomegaly [10], hereditary spherocytosis [6] and chronic lymphatic leukaemia [3] indicate a relationship between the pool size and the splenic size In reports of two groups of patients with miscellaneous disorders associated with splenomegaly a statistical relationship has been es-

tablished between pool size and spleen size [4, 12]. However, PETTIT *et al.* [8] who measured total splenic red cell content, found it to be less in the lympho-proliferative disorders as compared with the myelo-proliferative diseases and regarded this as being due to replacement of the splenic cords by lymphoid tumour tissue. Their findings have not been confirmed in this present study which shows no real difference between myelo-proliferative and lympho-proliferative disorders. The dependence of pool size and spleen size in various disease states during treatment or during progression of disease as shown in this work supports the concept of a splenic pool caused by splenic enlargement rather than by a specific pathological process. However, in some haemolytic anaemias the splenic pool may be disproportionately large [4].

The specific reservoir function of the spleen in many mammals was extensively investigated by physiologists 50 years ago [1] but the normal spleen in man has such a small blood content that a comparable role is negligible [9]. Nevertheless, the enormous volume of pooled blood in pathologically enlarged spleens indicates that a potential reservoir of blood exists which may be mobilised artificially by pharmacological agents [6, 10] and it is possible that this may occur under physiological conditions or as a response to haemorrhage. Many surgeons at splenectomy have developed the habit of squeezing the spleen before ligature of the splenic pedicle to give the patient an auto-transfusion. Hitherto the splenic pool has been regarded as being without specific beneficial function. Under stressful states it may confer some small, but positive, benefit.

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Serum B_{12} Binders in Vitamin B_{12} Deficiency

Relation of Serum B_{12} and Haemoglobin Levels with B_{12} Binders

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Abstract. In 74 adults with serum B_{12} ≤ 140 pg/ml with or without anaemia, unsaturated B_{12}-binding capacity (UBBC), total B_{12}-binding capacity and transcobalamin I and II (TC I, TC II) were determined using ^{57}Co-B_{12}. The results were statistically analysed in relation to severity of anaemia and vitamin B_{12} deficiency. In severe vitamin B_{12} deficiency, TC II was reduced and contributed to the reduction of UBBC. Greater unsaturation and moderate increase in total TC I were also seen. It is suggested that anaemia is a contributory factor in the moderate increase of TC I. On the basis of limited evidence presently available, it is argued that abnormal intestinal structure and function associated with severe vitamin B_{12} deficiency may result in impaired synthesis of TC II.

Key Words
Anaemia
Transcobalamins
Vitamin B_{12} binding
Vitamin B_{12} deficiency

Reduced total B_{12}-binding capacity (TBBC) has been noted in pernicious anaemia [1–4] and in nutritional B_{12} deficiency anaemia [1–5]. However, it has also been reported to be elevated [6]. Unsaturated B_{12}-binding capacity (UBBC) was either high [7] or equal to that of normal [2, 4]. It has also been observed that in B_{12} deficiency, the α-globulin B_{12} binder (TC I) has been elevated in its unsaturated form but total TC I (assuming all endogenous B_{12} is bound to TC I) was reduced [7, 8]. Changes in β-globulin B_{12} binder (TC II) and the role of anaemia in the metabolism of serum B_{12} binders (transcobalamins) have not been adequately studied. The present communication reports changes in transcobalamins in vitamin B_{12} deficiency with or without anaemia. It has been observed that in severe vitamin B_{12} deficiency, due to the reduction of TC II, UBBC and TBBC were reduced. Greater unsaturation of

TC I and limited increase in total TC I were also seen Presence of anaemia perhaps was a contributory factor in the increase of total TC I Release of TC I from liver and impaired synthesis of TC II due to changes in intestinal function and structure have been considered as possible mechanisms leading to the increase in TC I and reduction in TC II, respectively

Materials and Methods

A total of 74 adults were studied Screening procedures were based on following criteria of selection estimation of haemoglobin to ascertain anaemia, serum vitamin B_{12} levels of 140 pg/ml or less absence of any primary haematologic cardiac, renal and hepatic disorder Each of these patients was then subjected to detailed laboratory examination Standard haematologic techniques were employed Haemoglobin was estimated by the cyanmethaemoglobin method using a photoelectric colorimeter [9] Serum vitamin B_{12} using *Euglena gracilis Z* strain [10] and UBBC using ^{57}Co B_{12} (specific activity 1 μCi/μg) [11] were estimated TBBC was calculated therefrom The UBBC was further separated as TC I and TC II on rapid DEAE cellulose chromatography [12] using ^{57}Co B_{12} (specific activity 40–50 μCi/μg) Radioactivity in each fraction was counted in a scintillation counter The separation of TC I and TC II carried out in duplicate was reproducible The results were expressed as TC I and TC II, both as pg/ml as in UBBC and percent of UBBC. Based on the assumption that all endogenous B_{12} is bound to TC I in serum the addition of serum B_{12} and TC I (pg/ml as in UBBC) was expressed as total TC I (pg/ml) Estimation of serum iron [13] and unsaturated iron binding capacity [14] were done Serum folate [15] and red cell folate [16] levels were measured using *Lactobacillus casei* as the test organism Total serum proteins and albumin were also determined [17] The normal values of serum B_{12} binders are given in table I

Results

Anaemia was severe (haemoglobin 5 g% or less) in 19 (36%), moderate (haemoglobin 5 1–10 g%) in 21 (28 8%), and mild in 18 (24 7%) patients There were 15 (20 5%) non anaemic (Hb $\geq$ 12 g% in females and $\geq$ 13 g% in males) subjects with B_{12} deficiency Serum vitamin B_{12} was less than 50 pg/ml in 21 (28 3%), 50–99 pg/ml in 31 (41 8%) and 100 pg/ml or more in 22 (29 9%) subjects

Amongst these, iron deficiency (serum iron <40 μg/100 ml) and folate deficiency (serum folate less than 3 ng/ml) were seen in 17 of 86 (25 8%) and 16 of 69 (23 2%) patients respectively Red cell folate was low (< 100 ng/ml) in 9 of 66 (13 7%) patients Deficiency of protein

Table I. Range, mean and standard deviation of serum B_{12} and B_{12} binders in 35 normal subjects

B_{12} pg/ml	UBBC pg/ml	TBBC pg/ml	TC I % of UBBC	TC II % of UBBC	TC I pg/ml as in UBBC	TC II pg/ml as in UBBC	Total TC I* pg/ml
144-810	[illegible]-2,967	1,073-3,011	7.8-28.9	71.1-92.2	109-431	600-2,712	335-9[illegible]
281	1,7[illegible]	2,070	13.5	86.5	234	1,595	521
14[illegible]	453	419	5.6	5.6	94	437	134

* Assuming that all endogenous B_{12} is bound to TC I

Table II. Serum vitamin B_{12} in relation to UBBC

Serum B_{12} pg/ml	Number	UBBC pg/ml: <1,500 number	%	1,500-1,800 number	>1,800 number
<50	21	10	47.6	5	6
50-99	31	5	16.1 } 15.1	6	20
≥100	22	3	13.6 }	9	10
Total	74	18		20	36

Table III. Serum vitamin B_{12} in relation to TBBC

Serum B_{12} pg/ml	Number	TBBC pg/ml: <1,600 number	%	1,600-2,000 number	>2,000 number
<50	[illegible]	12	57.1	3	6
50-99	[illegible]	9	29.0 } [illegible]	[illegible]	[illegible]
≥100	[illegible]	6	27.3 }	8	8
Total	[illegible]	[illegible]		16	32

Table IV Serum vitamin B_{12} in relation to TC I

B_{12}	Number	TC I pg/ml as in UBBC <350 number	%	350–650 number	>650 number	% of UBBC <15 number	15–20 number	>20 number
	12	1	8 3	8	3	0	1	11
	8	2	25 0	5	1	1	1	6
	26	14	53 8	9	3	9	8	9
	46	17		22	7	10	10	26

Table V Serum vitamin B_{12} in relation to TC II

Serum B_{12} pg/ml	Number	TC II, pg/ml as in UBBC <900 number	%		900–1,500 number	>1,500 number
<50	12	7	58 3		3	2
50–79	8	1	12 5	8 8	1	6
≥80	28	2	7 7		11	13
Total	46	10			15	21

(serum albumin < 3 75 g%) was seen in 41 (60 3%), it was significant (serum albumin less than 3 25 g%) in 21 (30 8%) patients

Serum B_{12} Levels in Relation to Serum B_{12} Binders

Serum B_{12} in relation to UBBC and TBBC There is a clear evidence of an association of serum B_{12} and UBBC (table II) UBBC less than 1,300 pg/ml was seen in 47 6% of the patients with serum B_{12} less than 50 pg/ml as compared with 15 1% of those with serum B_{12} of 50 pg/ml or more This difference is statistically significant ($\chi^2_{(1)}$ = 6 966, $p <$ 0 01) A similar association of serum B_{12} was also seen with the TBBC (table III) TBBC less than 1,600 pg/ml was seen in 57 1% of the patients with serum B_{12} less than 50 pg ml as compared with 26 4% of those with serum B_{12} of 50 pg/ml or more This difference is statistically significant ($\chi^2_{(1)}$ = 4 277, $p <$ 0 05)

Table II Saturation of TC I in relation to total TC I

Saturation of TC I %	Number	Total TC I* pg/ml			
		<450		450–650	>650
		number	%	number	number
<15	23	6	26.1	9	8
15.1–30	15	8	53.3	7	0
>30	8	8	100	0	0
Total	46	22		16	8

* Assuming that all endogenous B_{12} is bound to TC I

Table III Haemoglobin levels in relation to UBBC

Haemoglobin g/100 ml	Number	UBBC pg/ml			
		<1,200		1,200–1,800	>1,800
		number	%	number	number
<8.0	19	7	36.8	4	8
8.1–10.0	21	4	19.0	7	10
>10	33	3	9.1	12	18
Total	73	14		23	36

Table VIII Haemoglobin levels in relation to TC I, total TC I and TC II

⊢ nl	Number	TC I >20% of UBBC number	%		TC I <350 pg ml as in UBBC number	%		Total TC I, pg/ml <450 number	%		TC II as in <900 number
	15	11	73 2	72 4	3	20	17.2	6	40	34 2	7
	14	10	71 4		2	14 3		4	28 5		2
	16	4	25 0		12	75		12	75		1
	45	25			17			22			10
		7 567			12 28			5 25			7 471
		<0 01			<0 001			<0 05			<0 0[illegible]

Proportion of patients with total TC I less than 450 pg/ml steadily increased *with the increase in percent of saturated TC I These differences* are statistically significant ($\chi^2_{(2)} = 13\ 264, p < 0\ 005$)

Haemoglobin Levels in Relation to Serum B_{12} Binders

Haemoglobin in relation to UBBC There is a clear evidence of an association of haemoglobin levels and UBBC (table VII) Proportions of patients with UBBC less than 1,200 pg/ml steadily decreased with the increase in haemoglobin levels These differences are statistically significant ($\chi^2_{(2)} = 5\ 9886, p < 0\ 05$)

Haemoglobin in relation to TC I (percent and pg/ml of UBBC), total TC I and TC II Patients with severe to moderate anaemia (haemoglobin 10 g% or less) had higher TC I (percent and pg/ml of UBBC) and total TC I as compared with those with mild or no anaemia (*haemoglobin more than 10 g%, table VIII) The association in all three instances* was statistically significant at 1-, 0 1- and 5-percent levels, respectively Low levels of TC II (less than 900 pg/ml as in UBBC) steadily decreased with the increase in haemoglobin levels (table VIII) These differences are statistically significant (p<0 05)

Serum B_{12} in relation to haemoglobin levels There is a clear evidence of an association of serum B_{12} and haemoglobin levels (ta-

Table IX Serum vitamin B_{12} in relation to haemoglobin levels

Serum B_{12} pg/ml	Number	Haemoglobin g/100 ml			
		5		5.1-10	10
		number	%	number	number
50	21	12	57.1	5	4
50-99	16	3	18.8	5	8
100	35	4	11.4	11	21
Total	73	19		21	33

ble IX). Proportion of patients with severe anaemia (haemoglobin 5 g% or less) steadily decreased with increase in serum B_{12}. These differences are statistically significant (χ^2 = 14.185, $p < 0.001$). In view of association of anaemia with serum B_{12} and serum B_{12} binders, it may be inferred that association between the latter two may be indirect, due to the associated anaemia.

A pair by pair analysis was done to find out the partial correlation coefficient to ascertain whether or not the relationship between any two factors was independent of the third factor. Relation of serum B_{12} with haemoglobin levels was significant (r 12 = 0.3475). Relation of serum B_{12} and haemoglobin levels with UBBC had low zero order partial correlation coefficients (r 13 = 0.1051, r 23 = 0.1461) and was not significant. Inverse relations of TC I (pg/ml as in UBBC) with serum B_{12} levels (r 13 = -0.3175) and with haemoglobin levels (r 23 = -0.4396) were significant, when haemoglobin was kept constant in former and serum B_{12} in the latter. The partial correlation coefficients for respective relationships were reduced (r 13.2 = -0.120, r 23.1 = -0.3236). Relation of TC I with haemoglobin levels, however, was still significant. Relation of TC I as percentage of UBBC was also examined in a similar manner with serum B_{12} and haemoglobin levels. Inverse relations of TC I (percent of UBBC) both with serum B_{12} (r 13 = -0.5702) and with haemoglobin levels (r 23 = -0.5645) were significant. When haemoglobin was kept constant in the former and serum B_{12} in the latter, the partial correlation coefficients for respective relationships were reduced (r 13.2 = -0.2481, r 23.1 = -0.4153) but still significant. Positive relations of TC II both with serum B_{12} (r 13 = 0.3594) and with haemoglobin levels

(r 23 = 0 3126) were significant However, when haemoglobin was kept constant in former and serum B_{12} in the latter, the partial correlation coefficients for respective relationships were reduced (r 13 2 = 0 2986, r 23 1 = 0 1481) Both these relationships were not significant but the former was highly suggestive

Discussion

Decreased TC levels (UBBC, TBBC) were found associated with severe vitamin B_{12} deficiency (tables II, III) TC II was also reduced (table V) and was responsible for this change Direct evidence of reduction of TC II in vitamin B_{12} deficiency has been noted in a small number of patients with pernicious anaemia [3, 18] which rose on vitamin B_{12} therapy [3] However, others [7] have reported normal TC II in these patients In non-anaemic healthy Indians, TC II was much higher [19] (mean 1,515±429 pg/ml) Thus, the reduced TC II levels would be interpreted as a major change Increase in TC I (percent of UBBC) seen in patients with severe B_{12} deficiency (table IV) was not only due to its greater unsaturation but was a result of increase in its total amount (table VI) (mean total TC I 480 5±136 pg/ml) [19] However, it was limited and did not affect the TBBC and UBBC levels substantially unlike TC II Greater unsaturation of TC I with reduction in total TC I has been observed in vitamin B_{12} deficiency in pernicious anaemia [7, 8 18], and it was not associated with increased TBBC Increase in TBBC in patients of pernicious anaemia, however, was noted on vitamin B_{12} therapy [2, 7]

Negative association of anaemia with TC I (pg/ml as in UBBC) was independent of accompanying B_{12} deficiency and, in fact, was responsible in great measure for the association of latter with TC I But the negative associations of TC I as percent of UBBC both with serum B_{12} levels and with anaemia were independent of each other However, the B_{12} deficiency was present in all patients and *per se* would result in increased unsaturation of TC I Therefore, it can be inferred that presence of anaemia perhaps was the main factor which led to absolute increase in TC I, and accompanying B_{12} deficiency was responsible for its unsaturation Granulocytes have been reported to be the site of production of TC I [20–24] It has been shown that marrow granulocyte reserve is reduced in vitamin B_{12} deficiency [25] However, severe vitamin B_{12}

deficiency is associated with extensive myeloid abnormalities in peripheral blood and bone marrow [26] but greater release of TC I from these atypical myeloid cells though unlikely, cannot be ruled out. Release of TC I from liver may be another possibility. Vitamin B_{12} is mainly stored in the liver [27] probably bound to a protein with β mobility [28]. The plasma B_{12} is a part of the major vitamin B_{12} pool in tissues [27] and TC I is responsible for transport of endogenous B_{12} out of the cell [29]. It may well be that, with prolonged deprivation of vitamin B_{12} and in presence of anaemia, the release of endogenous B_{12} into the circulation may be accompanied with unsaturated TC I and leads to its moderate increase in serum. Indirect evidence of such a mechanism is seen in cases with chronic liver disease (cirrhosis and kwashiorkor) [12, 30, 31]. Serum B_{12} and UBBC in these patients are elevated. An acute insult including fatty metamorphosis may result in temporary reduction of vitamin B_{12} in liver with its release into circulation [32-34]. The increased UBBC in some of these diseases [30] has been found mainly due to TC I. It has also been suggested that transcobalamins have properties similar to acute phase reactants [35]. However, it remains to be shown that anaemia *per se* can induce such a change.

Reduction of TC II associated with the severity of anaemia was greatly influenced by the accompanying vitamin B_{12} deficiency and in fact a true relation of TC II with anaemia was not seen. Mechanism of reduction of TC II seen here is not clear. The evidence in literature that vitamin B_{12} deficiency is associated with changes in structure and function of small intestine [36-39] may suggest that the vitamin B_{12} deficiency may affect the possible synthesis of TC II in the ileal mucosa. This postulation has earlier been made [40] but hitherto remains unsubstantiated. Malabsorption studies carried out in 5 patients with serum B_{12} < 50 pg/ml revealed presence of intestinal malabsorption in 3 patients. Five hour urinary excretion of D-xylose with 5 g of oral dose was less than 20% in 3 and abnormal fat excretion on fat balance test (100 g fat diet for 7 days) was seen in 2 patients (fat excretion 27.4 and 13.5 g/24 h). Schilling test done in 2 patients was normal (37%) in 1 and borderline (7%) in another. Jejunal biopsy in 2 patients with abnormal D-xylose test and steatorrhoea, however, was normal in 1 and showed partial villous atrophy in another. TC II in these 3 patients was very much reduced (241, 435 and 715 pg/ml).

Further studies are in progress to elucidate the exact of abnormalities of intestinal function and structure in the metabolism of TC II.

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Further studies are in progress to elucidate the exact role of abnormalities of intestinal function and structure in the metabolism of TC II.

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Effect of Corticosteroids on Eosinophil Leukocytes in Hypereosinophilic Syndromes

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Abstract. Corticosteroid treatment of patients with hypereosinophilic syndromes resulted in dissolution of the granules in the blood eosinophil leukocytes. Although the mechanism of these changes and their consequences are not known, they may represent a preparatory step in which the granule components in the cells to be marginated or removed from circulation are labilized.

Key Words
Corticosteroids
Electron microscopy
Eosinophil leukocytes
Eosinophilia

It is generally agreed that the function of the blood eosinophil leucocytes is to replenish the tissue reservoirs and that it is in the tissues that their functional activity takes place [7]. However, the significance of the well known response of blood eosinophils to ACTH (Thorn's test) or to corticosteroids is not clearly understood. When considering this eosinopenic response, a rapid and temporary removal [2] (margination or sequestration) of the blood eosinophils from the circulation eventually mediated by reticuloendothelial elements [4] may be taken into account. In the case of sustained corticosteroid administration, inhibition of the proliferation and of the discharge of bone marrow eosinophils may cause a more prolonged blood eosinopenia [7]. The fate of the cells removed from the circulation or prevented from leaving the bone marrow or possibly the spleen, the site of maturation of eosinophils in the rat, is unknown. Direct destruction of blood or of tissue eosinophils by corticosteroids, although considered possible by many investigators, seems not to be proven experimentally [5, 6].

The study of steroid effects on eosinophil blood leucocytes in normal subjects or experimental animals is limited by the small number of these cells in the circulation. On the other hand, our *in vitro* studies [9] in the rat indicated that incubation with corticosteroids might induce partial granule lysis of the eosinophils and intracellular liberation of the granule-bound peroxidase enzyme. These observations prompted us to examine the *in vivo* effects of corticosteroids on eosinophils in human subjects with a hypereosinophilic syndrome.

The corticosteroids were either infused intravenously in a single large dose, or given perorally in smaller doses over periods of 7–15 days The blood eosinophil leucocytes responded to this treatment with dissolution of their granules, an alteration leading to partial degranulation of the cells In the light of recent observations on eosinophil leucocyte kinetics [1, 10], these findings may elucidate some aspects of corticosteroid action on eosinophils.

Material and Methods

The examinations were carried out in 3 patients with a permanently high count of blood eosinophil leucocytes On the basis of their clinical findings, the primary disease was Loffler's pneumonia in one patient (female, aged 18 years) and eosinophilic endomyocarditis in another (female, aged 43 years) On corticosteroid treatment, both patients showed abrupt eosinopenia and apparent clinical improvement In a further patient (male, aged 44 years), the cause of eosinophilia was unknown He had episodes of eczematous dermatitis and slight complaints indicating an asthma like condition and showed hypersensitivity to many drugs This patinet did not react to corticosteroid treatment with eosinopenia and failed to show clinical improvement At 30 min, then at 2, 3, 12 and 24 h after the infusion of 100 mg of Diadreson F Aquosum®, the number of his blood eosinophils showed the following values 1,500, 1,570, 1 700, 2,200, and 2,300, respectively The preinfusion value was 1,600 Several examinations for helminthiasis gave negative results

Corticosteroid treatment was performed in 2 ways On one occasion, each patient was studied during a single intravenous infusion of 2–4 mg/kg body weight of Diadreson F Aquosum® (Organon) over a period of 30 min Furthermore, the blood eosinophil leucocytes were examined on several occasions in the course of peroral administration of prednisolone (Chemical Works of Gedeon Richter, Ltd., Budapest) for 5–15 days in a dose of 20–40 mg day The eosinophil leucocytes were examined before the beginning of treatment, at 30 min, at 2 4, 12 and 24 h after the infusion, or 1–5 days after the commencement of peroral treatment White blood cell and direct eosinophil cell counts were performed at these times, smears were taken and blood leucocytes prepared for electron microscopy by the method of WATANABE *et al* [12]

Results

Before corticosteroid treatment, the eosinophil leucocytes of the patients studied showed the typical appearance of mature or slightly immature cells both in the light and electron microscope. Slight deviations from the normal, assumed to represent alterations due to the increased production of eosinophils and possibly to the underlying disease, were also observed (fig 1). These changes, appearing slight when compared with those induced by corticosteroid treatment, are still under study and will be described elsewhere.

During the peroral corticosteroid treatment or after the infusion of a single large dose *of corticosteroids*, the blood eosinophil leucocytes showed only slight light microscopic changes. In cells sufficiently spread out, a few granules appeared more faintly stained than others, or 'empty' (fig 2). Degranulation of the eosinophils was not apparent at this time. Later, due to the very small number of eosinophils or to their complete disappearance, the alterations of the cells could not be judged with certainty.

In the electron microscope, the cells studied for 0.5–4 h after the infusion or in the first few days of peroral treatment showed severe alterations of their granules. In spite of the well-preserved granule membranes, the matrix of the granules was partially or completely dissolved. Around the granules there were electron-dense masses which, at higher magnification, showed to be made up of fine microvesicles (fig 3 and 4). Thus the granules appeared partially or completely 'empty'. At more advanced stages of dissolution of the matrix, there were only a few microvesicles attached to the crystalloids and to the granule membranes. In the 'empty' granules with or without crystalloids, membranous lamellar structures were also seen. Similar structures resembling myelin bodies were found also outside the granules (fig 5 and 6). In some of the eosinophils a few granules of intact structure were also observed. Immature granules without crystalloids seemed to be unaffected (fig 6). Other cytoplasmic organelles, mitochondria, cell membranes and nuclei appeared to be unaltered. At later stages, the few cells that were encountered contained but a few structures still recognizable as granules, only myelin body-like structures (fig 7).

These changes of the eosinophils were regularly observed in the 2 female patients who responded with eosinopenia to corticosteroids. However, in the third male patient who failed to show eosinopenia after the


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administration of corticosteroid, the eosinophils appeared intact after both types of treatment.

Discussion

The electron microscopic alterations of the blood eosinophil leucocytes seen in patients with permanently elevated levels of blood eosinophils indicate that, as a result of corticosteroid treatment, the granules are dissolved and their components released into the cytoplasm. The 'empty' appearance of some of the granule matrices does not prove that all of their components are dissolved, and it is uncertain which components of the granules have been discharged and which retained. Further studies with histochemical methods (for basic proteins, peroxidase, etc.) at the submicroscopic level are needed for a better understanding of these phenomena. It should be pointed out that the release of the granule substances into the cytoplasm does not necessarily mean release from the cells.

When considering the effect of corticosteroids on blood eosinophils it seems important to decide whether, under normal conditions of eosinophil production and blood eosinophil level, the cells would also be affected in the same way by corticosteroids or these alterations only occur

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Fig. 1. Electron micrograph of a blood eosinophil leucocyte before corticosteroid treatment. Most of the granules are intact. In a few, the density of the granule matrix is decreased, and loosening of its structure can be seen (arrows). These alterations may also be observed in the eosinophils of healthy subjects. Approx. [illegible]

Fig. 2. Blood eosinophil leucocyte 2 h after corticosteroid infusion. Focal degranulation (arrows) can be suspected. May-Grünwald-Giemsa staining. Approx. [illegible]

Fig. 3–[illegible]. Electron micrographs of the blood eosinophil leucocytes in corticosteroid-treated patients. *Fig. 3, 4.* Dissolution of the granule matrix and the appearance of [illegible] dense material around them can be seen. At higher magnification [illegible] a few attached to the surface of [illegible] and to the granule membranes (arrows). Approx. [illegible] and [illegible]. [illegible] Around the granules, membranous structures possibly representing the remnants of the dissolved granules can be seen. Approx. [illegible]. [illegible] and a few others [illegible] (arrows). The granule-lacking [illegible] are [illegible] in which only a few structures of granular origin can be observed. Approx. [illegible]

in certain forms of hypereosinophilic syndromes. The resistance of the blood eosinophils in one of our patients to corticosteroids is indicative of differences in sensitivity of the eosinophils to corticosteroids, as suggested earlier by DESCHIENS and POIRIER [3] and JEANNERET and ESSELLIER [8].

Unfortunately, we do not know the fate of the eosinophils marginated or sequestered during corticosteroid action, and the time sequence of the changes seen in the granules is also unknown. We do not know the site of action of corticosteroids either, whether circulating cells are affected or those marginated or sequestered (which in turn may reenter the circulation). Nevertheless, one might think that the changes of the blood eosinophils may represent a preparatory step for an easier release of granule components from the cells, either in the circulation or sojourning at the terminal vascular channels. On the other hand, it may be that granule lysis represents an early sign of cell destruction.

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Haematological Effects of Chronic Ethanol[1] Administrati and Folic Acid Deficiency in Mice

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Abstract To investigate the anaemia found in chronic alcoholics with liver damage model experiments in mice were performed lasting from 5 weeks up to 1 year Four groups were tested obtaining tap water (controls) 10% alcohol tap-water containing small amounts of methotrexate, or methotrexate plus 10% alcohol in tap water Alcohol or methotrexate alone induced no haematological changes. When combining both agents heav aemia developed indicating a synergistic effect of alcohol and folic acid def cy Only minimal changes could be observed in the livers of animals receivir cohol or alcohol plus methotrexate respectively

Key Words
Alcohol intoxication
Ethanol and haemopo
Folic acid deficiency
Methotrexate
Mouse haemopoiesis

Patients with alcoholic liver cirrhosis may show anaemia The quency of this complication ranges between 20 [1] and 80% [2] l observers report values around 50% [3-6] In about two thirds of t cases, anaemia is caused by bleeding from the gastrointestinal tract, the rest shows no signs of bleeding [7, 8]

The mechanisms of the anaemia not caused by bleeding are partly understood Erythrocyte life span was often found to be short [9, 10] and erythropoiesis showed a decreased normal or increased ue [7, 11] As no quantitative methods for the measurement of the of erythropoiesis were used, it has so far been impossible to decid which degree changes in erythrocyte destruction and/or erythropo contribute to the anaemia observed and which of the two mechanism the limiting factor This problem is at present under study in our lab tory using a quantitative method [12] for the estimation of erythropoi

[1] In this paper alcohol is used as a synonym for ethanol

Another question hitherto only partly solved is what causes this anaemia. Alcohol *per se* has been suspected. Only one experimental study reports haematotoxic effects of alcohol [13]; the experimental animals were dogs. ASCHKENASY *et al.* [14] found that rats react with anaemia when fed with wine; equivalent amounts of ethanol, however, were without effect. Finally, the combination of folic acid deficiency and alcohol intake has been shown to play a possible causative role in 3 cases of anaemia associated with chronic alcoholism and liver damage [15]. It is not established if this mechanism prevails more generally in this type of anaemia.

To obtain insight into the underlying causes of the anaemia associated with alcohol liver cirrhosis, we performed model studies in mice in one phase of the problem to investigate the possible aetiological roles of prolonged alcohol intake and folic acid deficiency on haematological parameters.

Materials and Methods

The animals used for the experiment were male NMRI mice with an initial weight of approximately 20 g. The animals were fed *ad lib.* [illegible] mouse pellets (Ab [illegible] No 21), Stockholm, Sweden, containing 27.5% protein and 10% fat) and tap-water containing different agents. The mice were separated into 4 experimental groups with respect to the following agents added to the drinking water: (1) [illegible] tap-water; (2) group E: 10% (v/v) ethanol in tap-water; (3) group M: methotrexate in tap-water (10 µg/4.0 ml); (4) group E+M: 10% (v/v) ethanol plus methotrexate (10 µg/3.2 ml) in tap-water.

Methotrexate [illegible] a folic acid antagonist [illegible] by Lederle (Cyanamid, Svenska AB) was used to induce folic acid deficiency. It was planned to give a dose of approximately 10 µg methotrexate per animal and day. The desired concentration of methotrexate was arrived at by first determining the volume of the [illegible] which the animals used per day. This [illegible] the amount consumed and the amount [illegible] a certain number of animals in a certain type of cage (8 mice per cage in our experiment). We arrived at a figure of 4.0 ml fluid [illegible] M and 3.2 ml [illegible] E+M.

[illegible] of [illegible] the [illegible] into erythrocytes of [illegible] (Radiochemical Centre, Amersham), [illegible] injected [illegible] 4 [illegible] before termination of the experiment was measured. [illegible] was determined in haemolysed blood in a [illegible] counter. The total amount of radioactivity [illegible] erythrocytes was [illegible] values for total haemoglobin [illegible] determined in our laboratory corrected for deviations of body weight and haemoglobin concentration in our experimental animals [1?]. At the termination of the experiment, the animals were anaesthetized by [illegible] of 3.4 mg pentobarbital sodium (Pentobarbital). Blood was obtained from the [illegible]. Haemoglobin concentration was determined by

the cyanhaemoglobin method in a Zeiss PMQ II spectrophotometer, haematocrit by the microhaematocrit method, and white and red cell number by counting in a Bürker chamber. Differential counts of the white cells were performed on smears stained with May Grunwald Giemsa. The concentration of folic acid in plasma was determined by the *Lactobacillus casei* assay. Body weight was determined on the living animal, the weights of liver, spleen and kidneys immediately after killing the animals.

Results

The experiments were extended over time periods from 5 weeks to 1 year.

Five weeks. The results from the 5-week experiment are presented in table I. Haemoglobin concentration, haematocrit, erythrocyte count and radioiron incorporation into erythrocytes decreased significantly in the E+M group. The anaemia observed was of moderate degree and showed a tendency (nonsignificant) towards macrocytosis and hyperchromicity. The white cell number decreased insignificantly in the E+M group.

Plasma folic acid concentration was not measurable in the M and E+M groups, the E group showed a moderately decreased value without statistical significance.

The white cell differential count did not differ significantly from controls. The body weight was significantly decreased in the E+M group. The liver weight increased significantly in the E group.

Eighteen weeks. Extending the experiment to 18 weeks brought about essentially the same type of effects but much more pronounced than in the 5-week experiment (table II). Haemoglobin concentration, haematocrit, erythrocyte count and radioiron incorporation into erythrocytes decreased heavily in the E+M group. The anaemia was somewhat macrocytic and hyperchromic but these changes were only of borderline significance ($p = 0.2–0.05$).

The white cell number was significantly decreased in the E+M group as was the eosinophil percentage in the differential count (results not shown).

In analogy with the 5-week study referred to, plasma folic acid concentration was not measurable in the M and E+M groups. Again, the E group showed a moderately decreased folic acid value, however, not being significant.

The body weight as well as liver and kidney weights were significantly decreased and spleen weight was increased in the E+M group. The

Table 1 Mean values (±SEM) in mice after drinking 10% ethanol, methotrexate solution or a combination of both for 5 weeks. The significance of the difference between the control and the test groups (analysis of variance) is expressed as p value

	Controls	Ethanol		Methotrexate		Ethanol plus methotrexate	
	n=5	n=5	p	n=5	p	n=5	p
Hb, g%	15.0 ±0.34	14.9 ±0.31	0.2	14.4 ±0.39	0.2	11.6 ±0.33	<0.001
Hct, %	47 ±0.6	46 ±0.7	0.2	45 ±1.4	0.2	36 ±1.4	<0.001
RBC, 10⁶ mm⁻³	10.1 ±0.16	10.4 ±1.40	0.2	9.9 ±0.30	0.2	7.4 ±0.30	<0.001
MCV, µm³	46 ±1.1	45 ±1.8	0.2	46 ±0.5	0.2	49 ±0.8	0.2-0.05
MCH, pg	14.9 ±0.47	14.4 ±0.63	0.2	14.5 ±0.18	0.2	15.8 ±0.15	0.2-0.05
MCHC, %	32.1 ±0.91	32.1 ±0.73	0.2	31.7 ±0.20	0.2	32.1 ±0.55	0.2
WBC, 10³ mm⁻³	6.5 ±1.47	5.6 ±1.31	0.2	6.4 ±0.53	0.2	4.4 ±1.01	0.2-0.05
PMN neutrophils, %	41.2 ±2.97	45.1 ±7.37	0.2	45.2 ±4.09	0.2	29.1 ±2.33	0.01-0.001
[illegible]	17 ±6.4	12 ±5.0	0.2-0.05	1	<0.001	<1	0.001
Body weight, g	21.9 ±0.93	21.3 ±1.06	0.2	28.5 ±0.43	>0.2	25.8 ±0.41	0.01-0.001
Liver, mg	1,319 ±116	1,597 ±49.7	0.05-0.01	1,340 ±21.1	0.2	1,333 ±25.7	0.2
Kidneys, mg	376 ±12.4	393 ±20.5	0.2	371 ±11.6	0.2	345 ±17.1	0.2-0.05
Spleen, mg	100 ±13.1	118 ±10.8	>0.2	100 ±5.8	0.2	119 ±11.5	>0.2

Table II Mean values (±SE) from mice after drinking 10% ethanol, methotrexate solution or a combination of both for *18 weeks* The significance of the difference between the control and the test groups (analysis of variance) is expressed as p value

	Controls	Ethanol		Methotrexate		Ethanol plus methotrexate	
	n=5	n=5	p	n=5	p	n=4	p
Hb, g%	13.1 ±0.26	13.2 ±0.30	>0.2	13.9 ±0.44	0.2–0.05	4.1 ±0.65	<0.001
Hct, %	42 ±1.2	43 ±0.6	>0.2	43 ±1.0	>0.2	14 ±1.9	<0.001
RBC, 10^6/mm^3	9.3 ±0.38	9.5 ±0.26	>0.2	9.4 ±0.24	>0.2	2.5 ±0.45	<0.001
MCV, μm^3	46 ±0.9	45 ±3.8	>0.2	46 ±1.4	>0.2	60 ±7.5	0.2–0.05
MCH, pg	14.1 ±0.43	13.9 ±0.26	>0.2	14.8 ±0.50	>0.2	17.1 ±2.24	0.2–0.05
MCHC, %	30.9 ±0.82	30.7 ±0.34	>0.2	32.0 ±0.36	>0.2	28.6 ±1.01	0.2–0.05
WBC, 10^3/mm^3	7.0 ±0.86	5.1 ±1.19	>0.2	5.7 ±0.85	>0.2	2.9 ±1.69	0.05–0.01
^{59}Fe incorporation, %	37.8 ±4.38	39.3 ±2.27	>0.2	34.4 ±3.27	>0.2	7.1 ±2.50	<0.001
Folic acid, ng/ml	73 ±25.8	58 ±8.3	>0.2	<1	<0.001	<1	<0.001
Body weight, g	37.1 ±0.79	38.3 ±0.84	>0.2	34.2 ±0.51	0.05–0.01	25.3 ±1.74	<0.001
Liver, mg	1,782±82.9	1,800±36.5	>0.2	1,538±40.9	0.05–0.01	1,184±7.8	0.01–0.001
Kidneys, mg	461 ±25.2	498 ±14.9	>0.2	485 ±8.7	>0.2	340 ±7.9	0.01–0.001
Spleen, mg	137 ±5.3	155 ±7.6	0.2–0.05	131 ±15.9	>0.2	251 ±144.7	0.05–0.01

Table 11.1 Means and (±SEM) values after drinking 10% ethanol, methotrexate solution or a combination of both for 1 year. The significance of the difference between the control and the test groups (analysis of variance) is expressed as p value

	Controls	Ethanol		Methotrexate		Ethanol plus methotrexate	
	n=5	n=5	p	n=4	p	n=5	p
Hb, g%	14.2 ±0.23	14.7 ±0.41	0.2	13.3 ±0.70	0.2	12.2 ±0.77	0.05-0.01
Hct, %	45 ±0.9	43 ±0.7	0.2-0.05	43 ±1.3	0.2-0.05	39 ±2.3	0.05-0.01
RBC, 10⁶ mm³	9.1 ±0.15	8.4 ±0.22	0.2	8.6 ±0.37	0.2	8.3 ±0.33	0.2
MCV, μm³	51 ±3.1	52 ±1.7	0.2	50 ±3.3	>0.2	47 ±1.0	0.2
MCH, pg	16.0 ±1.13	17.3 ±0.37	0.2	15.8 ±1.01	>0.2	14.6 ±0.52	0.2
MCHC, %	31.3 ±0.62	33.8 ±1.11	0.2-0.05	31.6 ±0.42	>0.2	31.1 ±0.52	0.2
WBC, 10³ mm³	1.9 ±0.36	3.7 ±0.31	0.01-0.001	3.8 ±0.16	0.2-0.05	3.8 ±0.41	0.01-0.001
Lymphocytes, %	47.7 ±4.41	49.2 ±2.58	0.2	37.8 ±1.62	0.2-0.05	39.3 ±2.43	0.2-0.05
Folate, ng/ml	23 ±2.3	38 ±4.2	0.05-0.01	<1	<0.001	1	<0.001
Body weight, g	400 ±11.3	414 ±14.3	>0.2	400 ±24.0	>0.2	409 ±10.2	0.2
Liver, mg	1,175±71.1	2,049±96.6	0.01-0.001	1,939±136.1	0.2-0.05	2,196±197.3	0.05-0.01
Kidneys, mg	554 ±29.9	654 ±24.1	0.05-0.01	631 ±32.7	0.2-0.05	727 ±27.0	0.01-0.001
*Spleen, mg	97	144		113		113	

body and the liver weights were significantly decreased in the M group In the E group, the spleen weight was significantly increased

One year The results of the 1 year study are shown in table III About 25% of the animals in the E+M group died before the 18th week of the study No macroscopic explanation could be found for their death but anaemia may have been one of the main reasons In the other groups, only sporadical deaths, mostly caused by biting were observed, they were equally distributed between the groups The results obtained after 1 year thus represent those of a selected group of survivors

In analogy to the experiments lasting 5 and 18 weeks described above, the E+M group showed a slight but significant anaemia The haemoglobin concentration and haematocrit were reduced Slight, but insignificant reductions of the haematocrit were also found in the E and M groups The index values were of the normochromic normocytic type Radioiron incorporation was slightly decreased in the M and E+M groups The change was of borderline significance

The white cell number was low in all experimental groups, but a significant, even lower number was noted in the control group As in the shorter experiments, folic acid concentration was not measurable in the M and E+M groups The control and E groups had substantially lower folic acid values than in the shorter experiments the E group had a significant, slightly higher value than the control group

In the E group, liver and kidney weights increased significantly Presumably, the spleen weight increased as well, however, pooling of organs did not allow calculation of the statistical significance No significant weight changes were observed in the M group The liver and kidney weight increased significantly in the E+M group Only very slight fatty changes could be found in the livers of the E and E+M groups after 1 year

Discussion

Induction of folic acid deficiency in mice by dietry means seems impossible because the intestinal bacteria of these animals synthetize folic acid [17] Therefore we had to produce the deficiency by other means, and methylaminopterin (methotrexate) was chosen for this induction The efficacy of the dose of methotrexate chosen to inhibit the physiologic effects of folic acid is indicated by the absence of measurable concen-

trations of folic acid in the plasma of the M and E+M groups (tables I–III), hence one criterion for studying the possible additive effects of folic acid deficiency and alcohol was fulfilled.

Furthermore, to be able to demonstrate any possible additive effect of alcohol it was desirable that the methotrexate dose was so low that the group receiving only methotrexate did not show haematological changes. Even this criterion was fulfilled, as still after 1 year of ingestion of methotrexate, haematological values were normal in the M group (table III).

As to the possible haematotoxic effects of alcohol alone, we can conclude that in our experiments, in contrast to those of Beard and Knott [13] in dogs, not even after 1 year of alcohol feeding, any changes could be noted. Beard and Knott gave their dogs 2–4 g/kg alcohol (33% v/v) by gastric tube after a 16–18 hour fast once a day for 8 weeks. Our mice got 16 g/kg alcohol (10% v/v) continuously as their only fluid supply. Even if we assume that half the amount was spilled, the dose still would correspond to 8 g/kg of pure ethanol per day. In spite of the fact that the alcohol dose given by us was 2–4 times higher than that of Beard and Knott, anaemia was induced in their dogs but not in our mice. One explanation for this obvious discrepancy must be sought in the great difference in the rate of alcohol breakdown between dog and mouse. Dogs eliminate alcohol at a rate of about 120 mg/kg/h whereas mice metabolize 600–800 mg/kg/h [18; Goldberg et al., in preparation]. In man this figure was found to be 80–110 mg/kg/h [19, 20]. Consequently the dose (2–4 g/kg/day) given by Beard and Knott [13] to their dogs, corresponding to 80–160 mg/kg/h, must have resulted in a considerable alcoholaemia (250–500 mg/100 ml). The dose given provides the dogs with 14–28 kcal/kg/day (7.1 kcal/g alcohol) which corresponds to about 40–50% of their basic metabolic need [18]. These calculations show that the alcohol must have had metabolic as well as pharmacologic effects in the dogs resulting in intoxication and in interference with food intake and, thus making haematotoxic effects possible.

Furthermore, the possible higher toxicity of 33% alcohol when given once a day in contrast to continuous intake of 10% alcohol, and the higher toxicity of alcohol when given by tube into an empty stomach, possibly causing local irritation, must be taken into account.

The alcohol breakdown, 5–7 times faster in mice, should result in much lower blood alcohol concentrations than those calculated for the dog, in spite of the dose given being 2–4 times higher than in the dogs.

Experiments recently performed by us in mice [GOLDBERG *et al*, in preparation] with exactly the same experimental design as in the studies reported here showed a mean daily blood alcohol concentration of 0 11 mg/100 ml (range 0 01–0 62 mg/100 ml) The morning values were significantly higher than the afternoon values, presumably caused by the higher physical activity and possibly even greater fluid consumption mice have during the night Addition of methotrexate did not change the blood alcohol values found The blood alcohol concentration found in our mice is thus much lower than that calculated for the dogs of BEARD and KNOTT [13]

The alcohol intake provides our mice with 60–110 kcal/kg/day which as mice have a much higher basic metabolic rate [18], comprises about 30–60% of their basal metabolic need Thus, the much higher alcohol doses given to our mice may evoke metabolic effects The low blood alcohol levels found and the results of actual experiments on the correlation between pharmacodynamic effects and blood alcohol levels [GOLDBERG and SKOG, in preparation] indicate the absence of pharmacologic effects at these doses These observations thus help to explain our negative haematologic findings

In contrast to these findings, when combining folic acid deficiency with alcohol (group E+M) anaemia was consistently induced This anaemia was moderate after 5 weeks but heavy after 18 weeks with a tendency towards a hyperchromic macrocytic picture Both times after 5 and 18 weeks radioiron incorporation into erythrocytes was decreased indicating that bone marrow depression is participating as a mechanism in causing this anaemia The role played by haemolysis cannot be evaluated as no parameters of haemolysis were studied

These findings lead to the conclusion that it seems to be the combined effect of alcohol and folic acid deficiency which causes the anaemia in our mice A similar mechanism in humans is suggested from findings reported by SULLIVAN and HERBERT [15] in 3 alcoholics with liver damage

Furthermore at least with respect to our experimental conditions we can conclude that a pronounced histologically manifest liver damage is not necessary to achieve the changes found That some effect on liver tissue may occur can be deduced from the inconstant changes in liver and spleen weight of the A group in some of the experiments The fact that alcoholics developing anaemia practically always show liver damage does not necessarily contradict our findings, as folic acid deficiency

is caused both by the metabolic and social effects of prolonged heavy drinking often requiring considerable time periods to develop. The metabolic effect of 150–200 g alcohol daily, an amount often encountered in alcoholics, gives a caloric intake of 1050–1,400 kcal comprising 50–70% of the caloric need in individuals with little muscular action. This metabolic effect makes the alcoholics to eat less, thus decreasing their folic acid intake. The slight reduction of folic acid concentration in the E group may be indicative of this. The decline in the social status chronic alcoholism often causes further decreases the intake of essential food stuffs and therewith folic acid. With increasing duration of abuse, the frequency of liver damage increases and may be concomitant but not necessarily causative to the development of anaemia. The combined effect of alcohol ingestion and folic acid deficiency indicates furthermore that alcohol *per se* must be haematotoxic even in our mice. This change, however, was not discovered in our experiments. The insignificant reduction of the haematocrit in E and M groups after 1 year may be indicative of this.

In contrast to the short term experiments, in the 1 year study, only slight anaemia is found. This may be explained by the observation that a substantial number of the animals from this group died early in the course of the experiment, leaving only those behind which were more resistant to the drugs.

The combination of alcohol with folic acid deficiency is toxic to leucopoiesis as well. This can be seen from the low leucocyte values in the E + M group in the 5- and 15 week experiments. In humans, leucocyte changes are inconsistent, all types of deviations have been reported [21, 22]. After 1 year, all mouse groups had low leucocyte values, those of the controls were significantly lower. We have no explanation for this finding.

The toxic effect of alcohol *per se* and of folic acid deficiency produced by the hydrate even finds its expression in reduction of body weight in the short time experiments. After 1 year, when most of the sensitive animals have died, no changes can be found.

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C. D. R. DUNN The Differentiation of Haemopoietic Stem Cells. Series Haematologica, vol 4, 1971

This review article provides a clear and comprehensive exposition of published work up to 1970 relating to 'colony forming units' (CFU). Studies of CFU s, where the colonies are produced *in vivo*, chiefly in the spleens of animals previously rendered aplastic by irradiation or cytotoxic drugs, are discussed in parallel with studies on CFU-c, where the colonies develop *in vitro* in culture media, usually containing agar gel together with 'colony stimulating factor' (CSF) provided by urine serum from various sources, or feeder layers of fetal cells.

The review is divided into three main sections, dealing in turn, for both CFU s and CFU-c, with *methods of assay and their interpretation, the patterns of differentiation* shown by colonies under normal conditions, and the influence of various potentially stimulatory or inhibitory agents such as erythropoietin, phytohaemagglutinin, cytotoxic drugs and hormones. The possible relationship of CFU s and CFU-c is then briefly discussed and the arguments in favour and against CFU being functional stem cells are summarised.

This is a fascinating field of study which holds great promise for the future. For the present, however, there remains uncertainty as to the cytological nature and potentialities of CFU, the extent to which CFU s and CFU-c, respectively, are multipotential or committed towards a specific cell line, and the factors influencing the differentiation pathway. The evidence on these questions, and others, is admirably marshalled in this review, which, with its bibliography of more than 200 references, provides an excellent introduction for cytologists and haematologists not yet familiar with this expanding research area. F. G. J. HAYHOE

T. ARENDS, G. BEMSKI and R. L. NAGEL (ed.) Genetical, Functional and Physical Studies of Hemoglobins. Proceedings of the 1st International Symposium on Hemoglobins, Caracas, December 1969. Karger, Basel 1971. VIII + 293 pp., 88 fig., 38 tab., sFr 80.–/US $ 19.20/DM 80.–/£ 8.80

Dieses Symposium war der gemeinsamen Arbeit einer grossen Zahl von Forschern gewidmet, die sich mit den verschiedenen Aspekten des Hämoglobins befassen. Im Vordergrund standen geographische, genetische, funktionelle und physikalisch-chemische Probleme. Aus den Referaten tritt uns das Hämoglobin mit seinen Modifikationen einmal mehr als eines der faszinierendsten Moleküle entgegen, das Kliniker, experimentelle Hämatologen, Biochemiker, Molekularbiologen, Genetiker und Anthropologen in gleicher Weise interessieren muss. Der Band ist in 3 Teile gegliedert, von denen jeder durch eine ausgezeichnete Übersicht über die Problemstellung eingeleitet wird: geographische Verteilung und genetische Probleme (H. LEHMANN, Cambridge), Struktur und Funktion (G. GUIDOTTI, Cambridge, Mass.) und physikalische Eigenschaften (M. GOUTERMAN, Seattle, Wash.). Im ersten Teil interessieren neben dem Vorkommen abnormer Hämoglobine in Südamerika vor allem die Konsequenzen der α-Kettenanomalien für Innenkörperbildung und Genlokalisation. Der zweite Teil bringt neuere Ergebnisse über die Baueinheiten und die

Konfiguration des Hämoglobin-Moleküls in ihren Beziehungen zu Aktivzentralstruktur, Oxygenierung, Oxydation (Methämoglobin) und Haptoglobinbindung. Besonders interessant sind dabei die Beziehungen zwischen Häm-Lokalisation und -Affinität zu seinen Liganden, Polypeptidkettenstrukturen und Sauerstoffbindung sowie die Rolle von Pyridoxalphosphat und Diphosphoglycerat. Aus dem dritten Teil erhellen die fruchtbaren Beiträge, die die Physik, ausgehend von den röntgenspektrographischen Untersuchungen von Perutz und Kendrew, zum Verständnis von Molekularbiologie und Funktion des Blutfarbstoffes zu leisten vermag. In die Geheimnisse der reversiblen Sauerstoffbindung und des Bohr-Effektes wird neues Licht geworfen. Im Vordergrund stehen Untersuchungen der elektromagnetischen Resonanz und der Spektrophotometrie. Zum Teil sind die Beiträge hoch spezialisiert und für den Mediziner nur noch am Rande verständlich (z.B. die Beiträge von M. Weissbluth über die Mössbauersche Absorption von Hämoglobinkristallen und von S. K. Dutt über den zirkulären Dichroismus und die optische Rotationsdispersion von Hämoglobin in Salzlösungen). Nach der Lektüre dieses drucktechnisch einwandfreien und mit sehr instruktiven Abbildungen ausgestatteten Buches kann man nur feststellen, dass die Voraussage von H. Lehmann in seiner Einleitung, das «Meeting would accumulate further bricks to a splendid monument of the science of human biology» sich erfüllt hat. Es ist zu wünschen, dass die folgenden «Proceedings» mit einem Sachverzeichnis ausgestattet werden, wie es für eine rasche Orientierung in einem so weit verzweigten Gebiet unentbehrlich ist.

H. Löffler, *Basel*

D. J. Weatherall and J. B. Clegg: The Thalassaemia Syndromes, 2nd ed. Blackwell, Oxford 1972. 374 pp., £ 7.00.

In 1965 Weatherall published the first edition of this standard book of reference. Since that time the basic knowledge on haemoglobin molecular pathology and disorders of haemoglobin synthesis in thalassaemias have increased considerably. As a consequence, the second edition has been completely rewritten and revised in collaboration with J. B. Clegg. The book is a modern complete review of the different thalassaemia syndromes and related disorders comprising genetics, genetic, molecular basis, pathogenesis, clinical picture and management of the resulting disease. In addition, the techniques of laboratory diagnosis and the buffer systems and stains are given as an appendix, followed by 40 pages of references. This book is essential for scientists and clinicians dealing with thalassaemia problems.

H. R. Marti, *Aarau*

P. C. Koller: The Role of Chromosomes in Cancer Biology. With a foreword by A. Haddow. Recent Results in Cancer Research, vol. 38. Ed. in chief P. Rentchnick. Springer, Berlin 1972. XII + 122 pp., 42 fig. DM 48.–; [illegible] ISBN [illegible]

Längst macht die Monographienreihe «Recent Results in Cancer Research» weit über Fachkreise hinaus von sich reden. Von bekannter Qualität, Ausstattung und Vollständigkeit ist auch wiederum die vorliegende Monographie. Sie stammt aus der Feder von P. C. Koller, dem Kenner der Materie. Das Buch ist übersichtlich in 11 Kapitel gegliedert, von denen [illegible]

kurzen Zusammenfassung versehen ist Ein Stichwortverzeichnis garantiert für eine rasche Orientierung Das Literaturverzeichnis umfasst zirka 200 Referenzen Das Buch ist anspruchsvoll, aber jederzeit verstandlich geschrieben Der Autor legt besonderes Gewicht auf eine klare Begriffsbestimmung und die experimentelle Dokumentation Indem theoretische Grundlagen und praktische Konsequenzen ver knüpft werden, profitieren Theoretiker, Praktiker und Lehrer sehr viel von dem kompendialen Inhalt

Das Buch führt den Anfanger zunachst in die Grundbegriffe der Karyologie, Zytogenetik, Nukleinsäurebiochemie und in zahlreiche Konzepte über die Zusammenhange zwischen Anomalien der chromosomalen Erbmasse und daraus resultie renden Krankheiten wie zum Beispiel Trisomien und geschlechtschromosomale Leiden ein Es erleichtert damit den Schritt von eher Bekanntem in das Hauptthema

Im wesentlichen wird sodann eingegangen auf die Haufigkeit und Art chromosomaler Aberrationen in malignen Geweben die Beziehungen zwischen Karyotyp *und Zytostatikaresistenz, Antigenitat, Enzymaktivitat, Hormonabhängigkeit sowie* biochemische Parameter von Tumoren, Verhalten der Chromosomen bei «spontanen» oder induzierten Tiertumoren, Chromosomenuntersuchungen in malignen Ergüssen und malignen Tumoren, «Marker» Chromosomen und ihre diagnostische und experimentelle Bedeutung unter besonderer Berücksichtigung des Philadelphia Chromosoms, Chromosomen in Präkanzerosen und in verschiedenen Phasen der Tumorentwicklung Chromosomanomalien und Krebserkrankungsrisiko, Zusam menhang virale Onkogenese und Chromosomenveränderungen, Virus DNA inte *griert in die Wirtszell DNA, Einfluss von Rontgenstrahlen und Zytostatika auf* Chromosomen, die Chromosomenanalyse zum Nachweis von Therapieschäden

Der Autor kommt zum Schluss dass – mit Ausnahme des ätiologisch mit der chronischen myeloischen Leukämie verbundenen Philadelphia Chromosoms – Chromosomenveränderungen in Tumoren sekundärer Natur, also Folge der ma lignen Veränderung der Zellen sind und sehr häufig jedoch sehr inkonstant, vor kommen, dass die vorherrschende Chromosomenveranderung nur ausnahmsweise für einen Tumor reprasentativ ist und je nach Wachstumsbedingungen des Tumors sehr wechselhaft ausgepragt sein kann was dem Tumor erlaubt, sich durch Vor schieben geeigneter Zellklone flexibel an seine wechselhafte Umgebung zu adaptie ren dass ein Tumor also ein Mosaik diverser Karyotypen darstellt und dass Zellen mit Chromosomenanomalien gegenüber Karzinogenen weniger resistent sind

Die Möglichkeiten, mittels Chromosomenanalyse Menschen mit erhöhtem Krebs risiko zu bestimmen Karzinogene als solche zu entlarven und Beziehungen zwischen Karyotyp und Antigenität von Tumoren aufzudecken, sichern der Karyologie ihre Zukunft G NAGEL, *Basel*

Acta Haemat. 49: 257-270 (1973)

Human Leukemia, Nucleic Acids and Viruses. Hypotheses and Perspectives[1]

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Abstract. Characterization of C type RNA viruses suggested that genetic and environmental factors are involved in the expression of the leukemia viruses. A reverse transcriptase in C type viruses, in human and animal leukemic leukocytes provides a mechanism by which an RNA tumoral virus may transform permanently a normal cell. However, it has been found the presence of reverse transcriptase in normal and different hematological conditions. The study of the genome of human leukemic cells is also related with the characterization of satellite DNAs, which are families of nucleotide sequences having different repetition frequencies. Experimental evidence obtained in animals make it possible that satellite DNA might be a point of insertion of viral genes in the cell to be transformed.

Key Words
Animal leukemia
Leukemia viruses
Nucleic acids in leukemia

Although the problem of etiopathogenesis of human leukemia is far from being resolved, spectacular progress has been made in recent years in the study of animal viruses and of viral and human nucleic acids. In this report these recent studies are emphasized and their relevance with reference to human leukemia is considered.

It has been known for several years that oncogenic RNA viruses play an important role in the development of tumors and particularly of leukemia in a variety of different kinds of animals. These C type RNA viruses have been isolated from several species [1, 2, 3, 4] and have been shown to be infective and to induce leukemic transformation in new born animals and in tissue culture. The name 'C type' virus derives from a morphological distinction of unknown structural basis, and later these viruses

[1] Conference held at the XIV International Congress of Hematology, São Paulo, Brazil 1972.

Table I Animal leukemias. C type RNA viruses most frequently studied

Species	Virus	Most frequent form
Avian leukemia	RPL 12 = BAI A = R = MC 29 RPL 12 = R = MC 29 = ES 4 BAI A	Lymphomatosis Erythroblastosis Myeloblastosis
Murine leukemia	GROSS passage A [28] GROSS passage X [29] KAPLAN [30] MOLONEY [31] RICH [32] HARVEY [33] TENNANT [34]	Lymphoid leukemia
	GRAFFI [35] JENKINS UPTON [36]	Myeloid leukemia
	FRIEND [37] RAUSCHER [38]	Erythroleukemia
Feline leukemia	JARRETT RICKARD [39, 40, 41]	Lymphoid leukemia
Guinea pig leukemia	OPLER GROSS [42 43]	Lymphoid leukemia

were alternatively called leukoviruses, oncorna viruses or, from TEMIN [15], RNA-DNA viruses Table I lists the viruses which have been isolated and which produce leukemia in animals which have been studied intensively such as chickens, mice and cats but which are rarely observed in natural conditions

As far as man is concerned, although several reports exist on the identification of viral particles in leukemia, there is at present much controversy on this matter, and it appears likely that viruses responsible for the development of leukemia in man have so far not yet been isolated with certainty In the last few years there has been a great deal of interest in the E B virus (a DNA virus) which seems to be responsible for the Burkitt lymphoma [5] Moreover, the E B virus seems also to be responsible for infectious mononucleosis [6, 7, 8] and ZUR HANSEN *et al* [9] have shown that the E B virus persists for a long time in reticular cells of patients who have suffered from mononucleosis It has also been suggested that cells from infectious mononucleosis exhibit in some *in vitro* and *in vivo* experiments a malignant behavior [10, 11]

Turning to murine leukemia it is known that in some inbred animal strains with high leukemia incidence, viruses are more easily demonstrated at the moment of birth or later in life, and some genetic factors may be involved in the expression of these leukemia viruses, which might also be influenced by environmental factors like radiation or carcinogenesis It has therefore been postulated that the genetic information for RNA tumor

Table II. Cesium chloride densities of human satellite DNAs [illegible] (from CORNEO *et al.* [25])

	Satellite			
	DNA I	DNA II	DNA III	DNA IV
Native in neutral CsCl	1.687	1.693	1.696	1.700
Heat-denatured in 1 × SSC	1.701	1.704	1.715	1.716
Renatured at a Cot = 0.1	1.694	1.696	1.703	1.706
Separated strands in alkaline CsCl	1.707 1.731	1.740 1.750	1.743 1.754	1.730 1.742
Density difference between complementary strands in alkaline CsCl	0.031	0.010	0.014	0.012
Separated strands in neutral CsCl	1.683 1.712	–	–	–
Reassociated amount %	0.5	2	1.5	2
Position in Ag⁺-Cs_2SO_4	light	heavy	light	light
Elution from a MAK column	late	early	late	early

viruses may be transmitted vertically in all vertebrates, and that a partial expression of this information could be responsible for leukemic transformation of normal cells. This may occur in the presence or in the absence of viral particles as it is more frequently observed in natural conditions. This last condition (the absence of virus) postulates the preexistance of viral genes in the genome of all vertebrates.

As a result of recent observations HUEBNER and TODARO [12] have proposed the oncogene hypothesis. This hypothesis states that the germ lines of all vertebrates as well as their somatic cells contain the DNA provirus for a type of C RNA tumor viruses. This 'information' can persist for several generations in [illegible] cell cultures, being maintained in an unexpressed form by repressors in normal cells. Various agents, and perhaps exogenously added viruses, may both transform cells by activating the endogenous [illegible] information and make viral specific products [illegible] the entire information of a [illegible] for leukemic transformation is [illegible] in the genome.

A very important step in this kind of investigation was taken independently two years ago by BALTIMORE [13] and by TEMIN and MIZUTANI [14] with the discovery of the reverse transcriptase, an RNA-dependent DNA polymerase, in tumor viruses and in tumoral tissues, and more recently, in human leukemic leukocytes This enzyme is able to make short pieces of double-stranded DNA on the copy of the tumor viral RNA, and this copy may be definitely inserted in the genome of the cell to be transformed, and to determine its tumoral transformation The relevance of this discovery is that it provides a mechanism by which an RNA-tumoral virus may permanently transform a normal cell This mechanism might be an alternative to the hypothesis of vertical transmission of viral genes and it is called the protovirus hypothesis by TEMIN [15] Following this hypothesis it has been proposed that in cells there might be regions of DNA that serve as a template for the synthesis of RNA and this RNA serves in its turn as a template for the synthesis of DNA that subsequently becomes integrated with cellular DNA By such means certain regions of DNA could be amplified, this being a model by which the differentiation of the embryonic cell might be explained

As regards leukomogenesis, if a C-type virus is introduced in a cell and additional process may occur, attributable to reverse transcriptase virus, that transfers the genetic information from the HMW-RNA of the virion to a piece of DNA which is later integrated in the nucleus of the infected cell

Although the oncogenic and the protovirus hypotheses do not exclude each other and they might in fact coexist, in naturally occurring leukemia, at least, the possibility of the insertion of viral genes in the cells to be transformed would appear now to be more likely after the discovery of the reverse transcriptase The discovery of reverse transcriptase is particularly interesting as far as human leukemia is concerned, because this enzyme can also be studied in human leukemic leukocytes Studies in this direction have in fact been carried out and this enzyme was found both [16] in normal and leukemic lymphocytes [17] Many problems concerning reverse transcriptase, however, are still to be resolved, for example the physical characterization of this enzyme, its function in neoplastic transformation, whether it distinguishes different templates, and whether some inhibitors may interfere in its enzymatic reaction It must also be stressed that reverse transcriptase is not unique to RNA tumor viruses but it is widespread in nature, having also been found in normal cells by COFFIN and TEMIN [18], SCOLNICK *et al* [19], BROWN and TOCCHINI-VALEN-

Table III. Buoyant density of human DNA CsCl density gradient centrifugation (from Cresto *et al.* [20])

	Main DNA, g/ml	Satellite DNA I, g/ml	Percent satellite DNA relative to main DNA
Bone marrow			
1 Normal	1.700	1.687	0.3
2 A.L.	1.700	1.686	1
3 A.L.	1.700	1.689	0.8
4 A.L.	1.700	1.685	0.8
5 A.L.	1.700	1.687	1
6 A.L.	1.700	1.687	0.8
7 A.L.	1.700	1.687	0.6
8 C.M.L.	1.700	1.689	0.6
9 C.L.L.	1.700	1.686	0.6
10 Myeloma	1.700	1.689	1.2
Leukocytes			
11 A.L.	1.700	1.687	1
12 A.L.	1.700	1.687	0.6
13 A.L.	1.700	1.687	0.5
14 C.M.L.	1.700	1.687	0.7
Lymphocytes	1.700	1.687	0.6
HeLa cells	1.700	1.687	1

test [20]. Researches are in progress in order to show if there are some differences in the reverse transcriptase activity between normal and neoplastic cells.

For several years Temin *et al.* have discussed the hypothesis that RNA-directed DNA synthesis plays a role in cellular differentiation, in antibody formation and gene amplification, and in recent years reverse transcriptase has come to play a role in cell transformation, but so far we do not know the full significance of the discovery of the reverse transcriptase. More recently reverse transcriptase and the 60–70 S RNA of oncogenic RNA viruses was detected at low concentration in the plasma of leukemic cats and mice [21]. In our laboratory we have investigated the presence of a reverse transcriptase activity in human sera from normal donors, from patients with leukemia (both acute and chronic), from patients with lymphoma, with megaloblastic anemia, aplastic anemia and

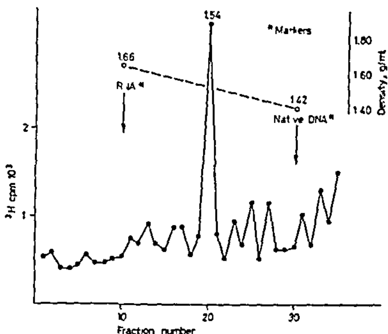


Fig 1 Cesium sulphate gradient analysis of the RNA [^3H] DNA complex from plasma concentrate of normal donors

with thalassemia minor The presence of a complex RNA plus reverse transcriptase in a pellet obtained from human sera is demonstrated by the detection of a labelled RNA-DNA hybrid in a Cs_2SO_4 gradient after incubation with radioactive deoxynucleotide triphosphate Following the SCHLOM and SPIEGELMAN technique [21] we have detected the RNA-^{3}HDNA hybrid in all sera of our patients Figures 1 and 2 show the behavior of the RNA-^{3}HDNA complex in cesium sulfate gradient analysis which is formed as a result of the reverse transcriptase reaction

Some simple experiments were performed to establish that the labelled peak is in that part of the gradient because of the hydrogen bonds linking the DNA strand with a large RNA molecule Figure 3 shows that mild denaturation after rupturing the hydrogen bonds with alkali can completely remove the newly synthetized DNA from the hybrid in which it was originally found, thus shifting the labelled peak from the hybrid region to the denatured DNA region in the Cs_2SO_4 gradient

The same conclusion can be reached by subjecting the pellet of the sera to the effect of the ribonuclease (fig 4) In this condition the formation of RNA-^{3}HDNA complex is not obtained and there can be seen only

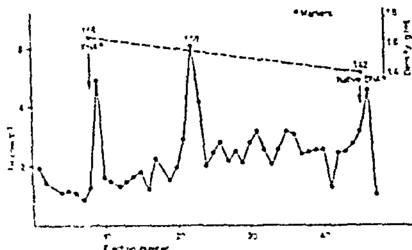


Fig. 2. Cesium sulphate gradient analysis of the RNA-[3H] DNA complex from plasma concentrate of a patient with blastic crisis of chronic myeloid leukemia

the formation of radioactive double-stranded DNA in the DNA region. Unfortunately, it is not easy to interpret the presence of the reverse transcriptase enzymes in non-infected cells and in normal sera. As is known from the experimental work of LEE HUANG and CAVALIERI [22], all DNA polymerases appear to have the capacity to use RNA as a template. The problem of gene amplification and other interesting points concerning the physical characterization of the DNA product of reverse transcriptase, are also related to the study of the genome of human leukemia cells.

Physicochemical studies on normal and leukemic DNA have shown that DNA contains families of nucleotide sequences having different repetition frequencies. The most highly repeated sequences are the satellite DNAs which have been isolated from the main DNA extracted in macromolecular form from normal and leukemic human cells by preparative caesium sulphate equilibrium density gradient centrifugation. Some experiments performed in our laboratory in the last few years have shown the presence of three satellites (Fig. 5). Then with fractionation experiments of human DNA on a MAK column we were able to demonstrate the presence of a fourth satellite (Fig. 6 and table III).

Although the biological role of satellite DNAs (which are randomly distributed in all the chromosomes) is not definitely known, it has been

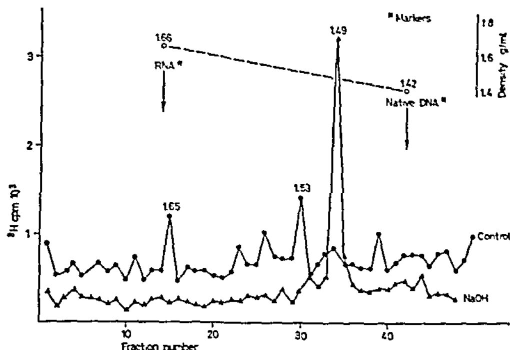


Fig 3 Cesium sulphate gradient analysis of the RNA [^{3}H] DNA complex from plasma concentrate of a patient with chronic lymphatic leukemia Effect of the denaturation with NaOH on the detection of RNA DNA complex ● = Control, ▲ = denatured

postulated that it may be inert genetic material originated in the past by saltatory replication (i e sudden replication of the genome) and available for genetic evolution A type of saltatory replication that might take place in a short time could be that induced by a widespread viral infection (lysogeny) and in particular some experimental evidence obtained in animals makes another saltatory replication possible, that is the demonstrated integration of viral DNA in the genome of the cell to be transformed However, studies carried out in our laboratory failed to reveal any difference between normal and leukemic satellite DNAs (table III) However, it must be said that the insertion in a DNA of a short piece of DNA replicated from a HMW-RNA by the reverse-transcriptase could not be easily detected

Another attractive hypothesis on the biological role of satellite DNAs is that they might be related to the organization of chromosomes If satellite DNAs are concerned with cell replication, their study might be particularly interesting in leukemic cells, in which the replication processes ap-

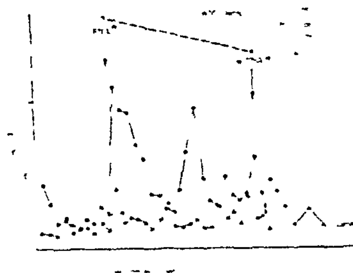


Fig. 4. C[illegible] pattern [illegible] of the RNA [illegible] DNA complex from plasma [illegible] of a patient with chronic myeloid leukemia. Effect of [illegible] on the [illegible] of the RNA DNA complex.

[illegible] be modified when compared to normal ones. By a new technique DNA RNA and DNA DNA [illegible] hybridization carried out on human chromosomes on a slide it has been shown [24] that at least a human [illegible] DNA ([illegible] 2) is located near the centromere of some human chromosomes ([illegible] 1, 9, 16). Work is in progress to localize other human [illegible] DNAs in the centromere of other chromosomes both in normal and leukemic [illegible].

[illegible] of nucleic [illegible] having lower [illegible] than the [illegible] DNAs have been isolated by [illegible] chromatography. Two well defined bands of these [illegible] DNAs in normal and leukemic tissues were described [25] and [illegible] of such [illegible] DNAs are [illegible].

Another [illegible] correlation between [illegible] and the presence of [illegible] form of DNA was found 5 years ago by [illegible] and [illegible] [illegible] [illegible] and [illegible] DNA from [illegible] and [illegible] DNA of [illegible] DNA [illegible]



Fig 5 Analytical ultracentrifugation of human native DNA in an Ag⁺ Cs_2SO_4 equilibrium density gradient I II III are satellite DNAs The preparation of the gradients and the condition of centrifugation are described in CORNEO *et al* [44] *analysis of the leukemia DNA is published in* CORNEO *et al* [45]

mechanism), has a circular structure, but HUDSON *et al* [27] found that in addition to this circular DNA, the mitochondria also contain DNA in a more complex form, such as dimers trimers, tetramers, and small chains DNA *in the form of circular dimer was found only in the cells of chronic* human leukemia (and in the cells of mouse myeloma) but the experimental data of CLAYTON and VINOGRAD [26] and HUDSON *et al* [27] require further confirmation

In conclusion, advances in knowledge regarding nucleic acids and cellular biological aspects of leukemia can be provided in the near future by these lines of research

1 Characterization of reverse transcriptase as regards it specificity in different species its capacity to distinguish different templates and its function during experimental leukemic transformation, might offer a promising approach to the understanding of the pathogenesis of leukemia

2 *Hybridization between DNA from leukemic cells and the RNA of* the C-type virus might resolve the problem if there is a transcription of the viral information in the genetic material of the somatic cells

3 Hybridization between total DNA or DNA fractions from leukemic cells and DNA from cells of infectious mononucleosis might give support to the hypothesis that infectious mononucleosis is a benign form of leukemia

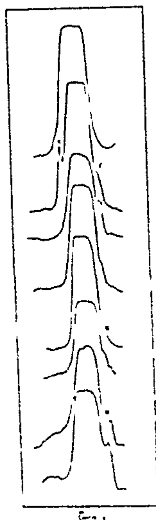


Fig. 8. Fractionation of human DNA [illegible] and centrifuged in Ag^+-Cs_2SO_4. I, II, III, IV are satellite DNAs (from Corneo et al. [illegible])

4. A further characterization of satellite and intermediate DNA in normal and leukemic cells might throw some light on the possible differences in the repeated nucleotide sequences of normal and leukemic nucleic acids. Concerning this point, it is interesting to remark that according to some authors, the repeated nucleotide sequences of cellular DNA might be a point of insertion for viral genome.

5. *In situ* hybridization experiments of repeated fractions (like satellites) to some of normal and leukemic cells might show a specific chromosomal distribution in leukemic conditions when compared with normal patterns.

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Some Characteristics of the Proliferative Activity of Erythroblasts in Untreated and Treated Acute Leukaemia[1]

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Abstract. In 7 cases of untreated and 2 cases of treated acute leukaemia (AL) the erythropoietic cell proliferation was studied by the combined method using Feulgen-cytophotometry and autoradiography after labeling with ³H-TdR *in vivo* (1 h after intravenous injection) or *in vitro*. A proliferation disturbance was observed, which was almost limited to the early polychromatic erythroblasts, consisting of an accumulation of diploid and unlabelled cells and a decreased proportion of cells in S. This proliferation defect was not present during complete remission. The results indicate the existence of non-cycling cells (G_0-cells) in the erythroblasts of AL, which may be responsible for ineffective erythropoiesis in this disease.

Key Words
Anaemia in leukaemia
Autoradiography
Cytophotometry
DNA synthesis
Erythropoiesis
Leukaemia

Anaemia, commonly seen in acute leukaemia (AL), has long been considered to result from invasion of the bone marrow by leukaemic blast cells with consequent displacement of the normal red cell precursors. However, an additional or alternative mechanism may be a reduced effective red cell production due to the inefficiency of erythropoiesis in acute erythroleukaemia and in acute granulocytic leukaemia [1, 4, 10]. *In vivo* labelling with tritiated thymidine (³H-TdR) in acute erythroleukaemia suggested a marked discrepancy between the proliferating capacity of the early erythropoietic precursor cell compartment (I_1, I_2) and the polychromatic one (I_3) and a strongly decreased influx into the non-proliferating cell compartment (I_4), indicating a premature

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exit from cell cycling and intramedullary cell death [2]. In order to gain further information about the proliferation pattern of the different erythropoietic cell types, and to demonstrate whether the decreased proliferation activity is reversible during remission, in the present study the erythropoietic cell proliferation in untreated and treated cases of AL was investigated by the combined application of cytophotometric determination of the DNA content and autoradiographic labelling with ^{3}H-TdR.

Material and Methods

Patients. Nine patients with AL were investigated. In 6 cases the bone marrow aspirates were studied before any treatment (No 1–6), in 1 case during the first relapse (No 7), and in 2 cases during complete remission (No 8 and 9) (table I). The cases 1–7 showed anaemia, thrombocytopaenia and/or granulocytopaenia at the time of the investigation. The proportion of erythropoietic cells in the bone marrow varied from 12 to 53%. In 1 case (No 6) erythropoietic hyperplasia with multinuclearity, karyorhexis figures, a strong PAS positive reaction and an increased number of sideroblasts were present, leading to the diagnosis of an acute erythroleukaemia. The cytological types of AL are given in table II.

General procedure. In 3 cases (No 1, 6 and 7) 0.1 μCi ^{3}H-TdR per kg body weight (specific activity 2 Ci mM) was given intravenously[1]; 1 h after the injection marrow was aspirated into a syringe containing 0.5 ml EDTA solution (1% Na_2EDTA in 0.9% NaCl). In 4 cases (No 2, 3, 4 and 5) the aspirate was incubated for 1 h with ^{3}H-TdR (2 μCi/ml, specific activity 2.0 Ci/mM) at room temperature. Smears were made from the marrow spicules and stained with Pappenheim stain. Optimal cell areas were marked with an object marker, and cells were photographed for subsequent localization for consecutive cytophotometry and autoradiography. Thereafter the stain was leached out by treatment with 50% ethanol and the smears were restained by the Feulgen method applying pararosanilin for Schiff's reagent (hydrolysis for 12 min in 1 N HCl at 60 °C, staining time 45 min).

Cytophotometry. For the determination of the DNA content a MPV Cytophotometer (Leitz, Germany) was used. Monochromatic light at 570 nm was employed, the area of the photometric field being 0.42 μm^2. The extinction (E) was measured at 10 points within the nucleus. The nuclear are (A) was determined from the nuclear diameters which were measured by an ocular micrometer. The relative DNA content (AU = arbitrary units) of individual nuclei was calculated from AU = E × A.

Autoradiography. Autoradiographs of the Feulgen stained smears were made by the dipping film technique using Ilford L 4 liquid emulsion. The smears were exposed for 20–30 days (*in vitro*) or 40–60 days (*in vivo*). The maximum back

[1] The patients were completely informed about the investigation procedure and agreed to it. They had a shortened life expectancy and all were treated by chemotherapeutic agents.

Table 1 [illegible] E:G = erythro:granulocytic ratio (normal range 0.2:1–0.4:1)

Case (age, sex)	Hb g/dl	RBC ×10¹²/l	Hct %	MCHC %	MCV fl	Reticulocytes ‰	WBC /µl	LBC %	Bone marrow: Ery %	[illegible] %	[illegible] %	total %	E:G	LBC %
1 [illegible]	11.7	3.91	34	31	89	70	23 000	89	4	9	8	21	1.9:1	48
2 [illegible]	7.6	2.52	24	30	92	25	1 900	0	2	44	5	51	3.2:1	26
3 [illegible]	8.6	1.05	28	21	43	11	10 900	1	3	37	14	54	2.6:1	22
4 [illegible]	9.6	2.41	27	42	106	8	900	4	3	31	8	42	7.6:1	33
5 [illegible]	6.3	1.96	19	33	95	1	400	43	10	1	0.2	11.2	8.4:1	80
6 [illegible]	8.4	2.8	27	30	66	4	4 900	15	9	25	31	65	2.9:1	13
7 [illegible]	13.1	3.36	39	33	116	57	12 800	71	1	14	14	29	1.9:1	45
8 [illegible]	11.4	3.53	34	33	96	15	4 300	0	4	20	20	44	0.9:1	0
9 [illegible]	10.9	3.46	37	32	107	21	2,700	1	6	19	26	51	1.3:1	0

ground was determined from lymphocytes and other diploid cells of the bone marrow. Cells showing more than 3–5 grains were considered as labelled.

Cytology. The classification of the erythropoietic cells was performed in panoptic stain. The following cell types were distinguished: basophilic erythroblasts (E_1–E_3), early polychromatic erythroblasts (E_4) and late polychromatic erythroblasts (E_5).[1] Apart from the cytoplasmic staining properties, cells showing a nuclear area smaller than 20 μm² were designated as E_5. The polynuclear cells or cells with other atypical nuclear formation observed in case 6 were not included into the study.

Results

By the technique described the cells examined can be attributed to the stages of the cell cycle. Diploid cells showing no labelling are assumed to be in the postmitotic resting period (G_1) or a non-proliferating cell pool (G_0), unlabelled tetraploid cells in the premitotic resting period (G_2) and the ³H-TdR-labelled cells in the DNA synthesis period (S). Unlabelled cells with a DNA content between the diploid and tetraploid level, which cannot be attributed to one of the resting periods, are designated as U-cells (U = unrecognizable [11]). For evaluation of the diploid standard (2c) the DNA values of the basophilic and early polychromatic cells in G_1 have been averaged. The mean tetraploid value (4c) was calculated from 2c.

In the *untreated cases of AL* the DNA content of basophilic erythroblasts was distributed from the diploid to the tetraploid level indicating the presence of a complete cell cycle in this cell type. A characteristic cytogram of one of these cases is given in figure 1. The fractions of basophilic erythroblasts in the different stages of the cell cycle are given in table II. Compared to the corresponding data of 4 normal humans, the proportion of cells in S is decreased in 2 of the 7 cases (cases 3 and 5), and an excess of G_1- or G_0-cells was observed in case 5 and of G_2 in case 3. *In the 5 other cases, the percentages of cells in G_1, S and G_2* were found within the normal range. In all but the cases the proportion of U-cells was higher than in normal erythropoiesis.

The early polychromatic erythroblasts, though ranging from 2c–4c too, showed a marked accumulation of diploid cells not labelled with ³H-TdR, and a reduction of the fraction of cells in the hyperdiploid range (fig. 1). The percentage distribution of cells in the different cell

[1] The morphological classification of the erythroblasts is described in a previous study [5]; the terms early and late polychromatic erythroblast were used instead of polychromatic and oxyphilic erythroblasts.

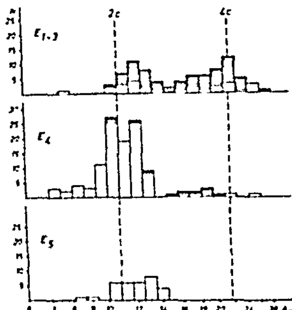


Fig. 2. Relative DNA content in arbitrary units (AU) and 3H-TdR labelling (shaded areas) of erythroblasts in untreated acute leukaemia (case 2). Semilogarithmic scale. N = number of cells. 2c = diploid standard. 4c = tetraploid standard. Basophilic ($E_{1,2}$), early polychromatic (E_4) and late polychromatic (E_5) erythroblasts.

cycle stages given in table II is showing a decreased proportion of cells in S from 7 to 29% compared to 55-75% in the normal erythropoiesis, and an elevation of cells in G_1 or G_2 from 43 to 87% (normal 8-30%) in all 7 cases. In some cases it was difficult to attribute the cells to the different cell cycle stages, especially in case 7, and high percentages of [illegible] were present. In all cases, however, the results of cytophotometry were comparable to those obtained by autoradiography. In the cases showing a relatively high number of hyperdiploid cells a considerably high labelling index, and in cases showing few hyperdiploid cells a considerably low labelling index was evaluated. In the late polychromatic erythroblasts a diploid DNA content and no autoradiographic labelling was observed as seen in normal erythropoiesis (table II).

In complete remission of AL (cases 8 and 9) the DNA values of the basophilic as well as of the early polychromatic erythroblasts were di-

ground was determined from lymphocytes and other diploid cells of the bone marrow. Cells showing more than 3–5 grains were considered as labelled.

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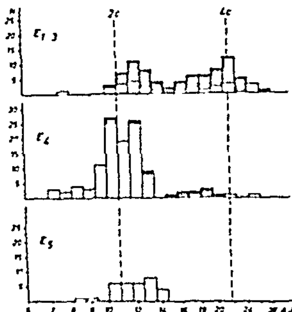


Fig. 1. Relative DNA content in arbitrary units (AU) and 3H-TdR labelling (shaded areas) of erythroblasts in untreated acute leukaemia (case 2). Semilogarithmic scale. N = number of cells. 2c = diploid standard, 4c = tetraploid standard. Basophilic (E_{1-3}), early polychromatic (E_4) and late polychromatic (E_5) erythroblasts.

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In cases of remission of AL (cases 8 and 9) the DNA values of the basophilic as well as of the early polychromatic erythroblasts were di-

e II Percentage distribution of erythroblasts in the different DNA synthesis stages (G_1, S, ed and treated acute leukaemia n = Number of cells, U = unrecognizable cells (see text) M values (4 cases) from QUEISSER *et al* [5]

Diagnosis	Basophilic erythroblasts					Early polychromatic erythroblasts					Late polychr erythroblasts		
	n	G_1	S	G_2	U	n	G_1	S	G_2	U	n	G_1	S
Untreated AML	129	19	67	9	5	131	50	24	12	14	40	100	(
Untreated AML	81	16	69	11	4	115	87	10	2	1	32	100	(
Untreated AUL	128	30	22	39	9	125	43	24	27	6	41	98	(
Untreated AML	115	15	74	9	2	119	61	29	8	2	42	100	(
Untreated AMML	127	49	37	10	4	104	80	7	6	7	9	100	(
Untreated AEL	116	21	76	3	0	151	67	26	4	3	22	100	(
1st relapse AML	119	21	55	12	12	144	62	15	23		25	96	(
Complete Remission ALL	94	14	81	5	0	105	31	59	8	2	45	100	(
Complete Remission AUL	109	25	54	13	8	92	27	52	15	6	72	100	(
n normal values ises)		28 (25–37)	63 (51–70)	8 (5–11)	1 (0–1)		19 (8–30)	65 (55–75)	15 (5–26)	1 (0–1)		96 (95–100)	(6

tributed from 2c to 4c and did not show an accumulation at the dipl level as seen in untreated cases of AL The cytogram of one of the t cases is given in figure 2 The percentages of cells in the different c cycle stages were ranging within the normal limit (table II)

Discussion

Impairment of red cell production in AL is present not only in ca showing a complete blast cell infiltration of the bone marrow and d

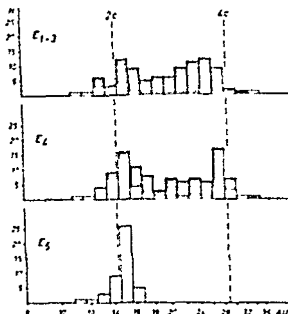


Fig. 2. Relative DNA content in arbitrary units (AU) and 3H-TdR labeling (shaded areas) of erythroblasts in acute leukaemia during complete remission (case I).

placement of the normal erythropoietic precursors, but also in acute erythroleukaemia which is characterized by hyperplasia of the erythropoietic cell line, in cases of preleukaemia and smouldering acute leukaemia [1, 9] showing a very low or moderate number of leukaemic blast cells in the marrow. Consequently, the anaemia in certain cases of AL can rather be explained by a dysfunction than by the diminution or absence of the tissue in the erythropoietic proliferation-maturation compartment. It was the objective of the present investigation to elucidate the pathogenetic mechanism of such an apparently ineffective erythropoiesis in AL.

The results suggest a proliferation disturbance of the erythropoietic cell line, consisting of an accumulation of diploid cells labelled with 3H-TdR and concomitantly a decreased proportion of cells in S. This defect was a most [illegible] early polychromatic erythroblasts. In the one case of erythraemic myelosis studied by Wickramasinghe et al. [10]

similar changes were observed in both the basophilic and early polychromatic cells, being less marked than in our study In 9 cases of AL, which were investigated by cytophotometry and *in vitro* autoradiography with ^{3}H-TdR by HUBER *et al* [3, 4], the number of erythroblasts in DNA synthesis was significantly reduced in comparison with normal subjects

From these results a prolonged average generation time of erythroblasts has been suggested However, a normal, a shortened as well as a prolonged DNA synthesis time was observed in cases of acute erythroleukaemia by other methods [2] Therefore, the decrease of the ^{3}H-TdR labelling index may be secondary to the accumulation of diploid and unlabelled cells and it seems to be more likely that the proliferation defect in the erythropoietic cell line is localized mainly within the non-proliferating cell pool

It cannot be decided whether these diploid cells are in cycle (G_1) or out of cycle (G_0) If they were in cycle, a marked prolongation of the post-mitotic resting period would have to be postulated Autoradiographic studies of the erythroblasts in acute erythroleukaemia after *in vivo* labelling with ^{3}H-TdR suggest that the efflux of cells of the proliferating compartments into the non-proliferating compartment E_3 is markedly reduced [2] From these observations the most likely explanation of our findings may be that a relatively small proportion of the diploid cells is in cycle, whereas most of them are out of cycle The out-of-cycle cells may mature directly to E_3 without proliferation or may underlie an intramedullary cell death

A similar proliferation defect has also been observed in preleukaemic acute leukaemia [8] and in other conditions showing ineffective erythropoiesis as thalassaemia [7, 12] and congenital dyserythropoietic anaemia type II [6] Therefore ineffective erythropoiesis showing an accumulation of diploid resting cells in the early polychromatic cell compartment is regarded as a common patho-physiological mechanism, resting on quite different aetiological factors

In case 3 a high proportion of G_2-cells in both the basophilic and early polychromatic erythroblasts was observed, and in most cases the percentages of U-cells were higher than in normal subjects These changes may be the result of pathological mitoses terminating in cells showing an aneuploid DNA content or of DNA synthesis arrest at the end of the S phase as it has been observed in vitamin B_{12} or folate deficiency anaemia [11, 13] From the results obtained in the present investigation it cannot be decided, whether an exogenous or endogenous

vitamin deficiency is playing an additional role for the development of ineffective erythropoiesis in AL.

The results in the 2 cases studied during complete remission demonstrate a high proportion of cells in S and a considerably lower one in G_1, as observed in normal subjects. The fact that the proliferation defect is not present in complete remission is due to the absence of anaemia during this stage. This observation suggests that the proliferation disturbance described may be an important factor for ineffective erythropoiesis in AL.

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Transplantable Myeloid Rat Leukaemia Induced by 7,12-Dimethylbenz(a)anthracene

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Abstract. With repeated intravenous administrations of DMBA transplantable myeloid rat leukaemia has been induced. In the course of transplantations 2 forms have developed: (1) the subacute form maintained with liver and spleen cell suspensions yielding a true leukaemic blood picture; (2) the ascites tumour form without leukaemic blood picture. The subacute form of leukaemia following serial transplantation has kept its original character.

Key Words
Dimethylbenz-anthracene
Rat leukaemia
Transplantable myeloid leukaemia

Several investigators have successfully induced leukaemia by oral or intravenous administration of 7,12-dimethylbenz(a)anthracene (DMBA) or other cyclic aromatic hydrocarbons [4, 6, 11] in the rat. The induced leukaemias are mainly of the lymphoid type. Leukaemia of the myeloid type, rather few in number, usually cannot be transplanted for a long time. The present study deals with the histological, haematological and biological features of a transplantable myeloid rat leukaemia.

Materials and Methods

For the leukaemia groups [illegible] rats weighing between [illegible] and [illegible] g were used, of which 25 were WOP H(Ohs) inbred females and 25 Wistar H(Ohs) outbred (12 females and 13 males).

DMBA [illegible] was suspended in [illegible] which was injected on 4 occasions [illegible] The animals were kept under [illegible] in groups of 15–25 [illegible] in metal cages. They were provided a commercial [illegible] and water ad libitum.

Haematological examinations From the blood obtained by puncturing the retro-orbital plexus WBC RBC and thrombocyte counts were determined regularly The blood smears were stained according to May Grünwald Giemsa (MGG) From the femur of the killed animals we obtained bone marrow smears and sedimented bone marrow performed in a sedimentation chamber [1 3] which were stained by MGG too The results of the 2 methods were in accordance with each other but the individual cells were easier to identify in the preparations settled in the sedimentation chamber In the peripheral blood bone marrow and ascites the following reactions were performed peroxydase in part after SATO SEKIYE in part after LEDER [7] non specific esterase according to BURSTONE [9] with naphthol AS D chloroacetate substrate [10] alkaline phosphatase according to GÖMÖRI and TAKAMATSU as well as Sudan black B staining after SHEEHAN and STOREY [12]

Histological examinations The organs of the primary and transplanted leukaemic animals (liver spleen lymph nodes thymus bone marrow, lungs kidneys and adrenals) were embedded and the sections stained with haematoxylin eosin (HE) and occasionally PAS reaction was also performed

Transplantation studies The blood bone marrow suspension liver suspension and mixed liver spleen suspension of the primary leukaemic animals were injected intraperitoneally intravenously subcutaneously and on some occasions intracranially into 2 to 4-day-old rats (sucklings of the same litters) and into 2 to 4 week old rats, each group consisting of 10 animals

Results

Of the 50 rats having received intravenous DMBA leukaemia developed in 5 animals viz in 3 of the 25 inbred rats and in 2 of the 25 outbred rats Primary leukaemia became manifest between the 5th and 9th month following the initial DMBA injection The condition of the animals deteriorated with stimultaneous weight loss and enlargement of the spleen to palpability WBC count at this time rose to values between 120 000 and 260 000 mm³ In the blood smear myeloid elements at all stages of maturation were found (fig 1a) in 80% they were peroxydase positive (fig 1b) At autopsy besides the spleen (weighing between 6 and 16 g instead of about 1 g in the untreated rats) all the lymph nodes were moderately enlarged Histological examination revealed that the cells participating in the infiltration of the bone marrow liver, spleen lymph nodes did not correspond either to reticular or lymphoid cells but to mature and immature myeloid elements similar to those observed in the blood They were polygonal with clear and eosinophilic cytoplasm Their nuclei were round bean shaped ring shaped or lobulated with several nucleoli (fig 2) Among them phagocytizing cells showing PAS positivity could be detected Under low magnification these cells looked like light

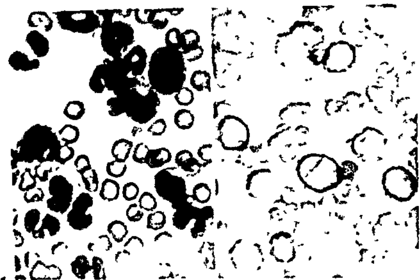


Fig. 1. Peripheral blood from DMBA-treated leukaemic rat. a May-Grünwald-Giemsa staining. b Peroxydase reaction. × 450

spots in the dark, monotonous cellular infiltration similar to the 'starry sky' structure of Burkitt's lymphoma [13]. In the bone marrow normal myelopoesis or erythropoesis could not be observed, but only the above-described types of cells (Fig. 2). The original cellular substance was indicated only by occasional megakaryocytes. Infiltration in the liver was found to take place around the central veins and in the portal area, hardly extending, however, to the hepatic lobules. The pulp of the spleen was infiltrated but the follicles were spared. The lymph nodes displayed a 'starry sky' structure. The capsule and even the adjacent fatty tissue showed leukaemic infiltration.

The leukaemia was successfully transplanted from the primary leukaemic rats. After some transplant generations we kept only a single line [illegible] by using the following procedures: (1) combination of liver and spleen cell suspension, (2) liver cell suspension and (3) [illegible] blood. These were all given [illegible] 2 [illegible] transplantation.

All [illegible] were [illegible] the 180–185 passage and it was found that leukaemia [illegible]

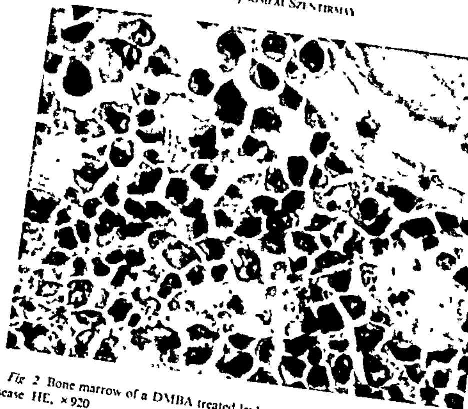


Fig. 2 Bone marrow of a DMBA treated leukaemic rat in an advanced phase of th[e] disease. HE, ×920

Table 1 Comparison of cells in the peripheral blood of normal, primary leukaemic and transplanted leukaemic rats (500 white blood cells counted in MGG stained smears)

Peripheral blood	Normal rats %±SD (n=20)	Primary leukaemic rats % (n=5)	Leukaemic rats from the 18th to 22nd passages %±SD (n=20)
Blast, promyelocyte	0	38	29.1 ± 5.4
Myelocyte, metamyelocyte	0	19	23.5 ± 3.8
Juvenile metamyelocyte Polymorphonuclear	31.4 ± 5.4	28	21.1 ± 2.8
Lymphocyte	69.6 ± 5.4	16	21.0 ± 2.9
Undefinable	0	0	5.3 ± 3.0
Nucleated red blood cell		sporadically 1	10/100 WBC

Table II. Summary of the cytochemical reactions of the peripheral blood and bone marrow cells in normal rats and in rats after serial transplantations (groups of 20–20 rats)

Peripheral blood cytochemical analyses	normal rats %±SD	leukaemic rats 18th 22nd passages %±SD	Bone marrow normal rats %±SD	Bone marrow leukaemic rats 18th 22nd passages %±SD
Peroxidase positive	32.5±10.1	49.8±4.6	45.5±1.4	30.0±2.9
Sudan Black B positive	29.3±4.1	68.4±3.1	41.7±1.4	57.3±1.5
Naphthyl esterase positive	16.0±9.6	9.6±5.9	41.3±5.9	32.0±7.1
Alkaline phosphatase positive	31.5±9.0	1.7±2.8	37.6±7.0	6.4±3.2
[illegible] with NMGG staining	31.0±7.1	71.0±3.5	61.0±2.2	85.0±2.7

haematological and histological pictures. The best takes, however (98%) could be achieved by the simultaneous administration of liver and spleen cell suspensions into 2- to 4-day-old animals. Thus, from the 15th passage on the leukaemia was maintained in the latter way only. In the following this form of the disease will be denoted the 'subacute form' in contrast to the 'acute form' to be discussed later.

At present we are at the 35th transplant generation of the subacute form. Average life span is 34±9 days, calculated from the life span of 1251 rats. Spontaneous regression has not been observed. The leukaemic blood picture appears between the 12th and 20th days. Before death WBC count varies between 70000 and 260000/mm³. RBC and thrombocyte counts decrease only at extremely high WBC counts. When comparing the blood picture after several transplantations to that of primary leukaemic animals, it is noteworthy that the blood picture keeps its myeloid character and is extremely variable (Fig. 3a). The ratio of the more mature cells (metamyelocytes, polymorphonuclears) when compared to primary leukaemia decreases somewhat and cells not identifiable with certainty appear in some percentage. The lymphocytes are normal both in the primary and transplanted leukaemic animals even after several transplantations (15). [illegible] on the basis of cytochemical re-



Fig. 3. Haemopoiesis in a rat inoculated with liver and spleen suspension i.p. 18th transplant generation. *a* Peripheral blood: 180 000 WBC/mm³. May-Grünwald-Giemsa staining. ×660. *b* Sedimented bone marrow cells: 95% blast cells. May-Grünwald-Giemsa staining. ×900.

actions is given in table II which shows that peroxydase positivity together with Sudan black B positivity were found to be much higher than normal. Non specific esterase and alkaline phosphatase activity are reduced. The bone marrow cells of leukaemic animals stained after MGG are almost uniformly big cells with lively blue cytoplasms (fig. 3b). In the nucleus they have fine chromatin and one or two nucleoli. Granulation can not be detected even in the cells with perforated nuclei which occur in 2% of the cells. Their peroxydase and Sudan black B positivity, however, stand for the myeloid character of these cells. In addition to these blast like cells mature granulocytes with ring shaped nuclei occur in 1–2%. The erythroid elements at various stages of maturation can be found in 3.5% in contrast to the 39% found in the bone marrow of the normal rat. It has been shown that positivity but also the intensity of non specific esterase and alkaline phosphatase activity are reduced in about half of the cells [9, 10].

In the course of transplantations the macroscopic picture has changed (fig. 4) inasmuch as the lymph nodes particularly in the mesentery show a marked increase and a greenish colour. The spleen keeps to be considerably enlarged in average 3.3 ± 1.1 g/100 g body weight in contrast to the spleen of normal rats in which the average spleen weight is



Fig. 4. [illegible] leukaemia, liver and spleen [illegible] of the 26th [illegible] on the [illegible] day [illegible]

0.46 ± 0.13 g/100 g body weight). Also the liver is enlarged. After several [illegible] (22nd passage) the type of the organ [illegible] as compared [illegible] primary leukaemia remains [illegible] unchanged. Neither does the [illegible] change. [illegible] appear in the lungs, peri[illegible] and [illegible] the renal [illegible] in the liver may [illegible] The [illegible] does not participate in the process.

The acute ascitic form In the animals inoculated with hepatic and splenic cells as described above, also ascites may develop and at the same time the blood picture is leukaemic In the ascites, occasionally developing in this way, the cells are of a myeloid character, and they are at different stages of maturation similar to those found in the blood of the same animals These ascitic cells show peroxydase positivity in a considerable percentage These ascitic cells can be transplanted i p into adult rats (120–150 g) as well After 3 or 4 transplantations however, the cells became dedifferentiated and peroxydase negative The acute ascitic form has been derived from such an ascites bearing leukaemic animal This ascites tumour is maintained in rats weighing 120–150 g with the i p inoculation of 10 million tumour cells per rat At present we are at the 170th transfer generation The ascitic form does not produce either a characteristic blood picture or metastases into the organs

Discussion

We have not found spontaneous leukaemia either in our Wistar outbred stock, raised at our Institute for 16 years, or in the WOP inbred strain The incidence of other spontaneous tumours is very low, too, developing occasionally at a very old age (fibroma-like tumours) but not exceeding 0 5% This finding might explain the fact that with 4 subsequent intravenous administrations of DMBA, only in 10% of the animals could leukaemia be induced, this leukaemia proved to be of the myeloid type

The results of blood and histological examinations all stand for the myeloid character of the leukaemia, viz. the type of the organ-infiltrating cells and the character of these infiltrations Cytochemical studies of haematopoiesis reveal some resemblance to the picture of human chronic myeloid leukaemia like, e g the rise in the number of peroxydase and Sudan black B positive cells and the reduction in alkaline phosphatase positivity in these same cells Non-specific esterase activity is strongly reduced in the peripheral blood, while in the bone marrow, it diminishes only slightly as compared to the blood and bone marrow smears obtained from normal animals just as it occurs in human acute myeloid leukaemias, too [8 14]

The bone marrow cells in about 65% without any granulation stained by MGG seem to be blasts, some of them, however, show certain signs of differentiation e g perforated nuclei which characterize myeloid cells in rats during the process of maturation from promyelocytes on only [1] The peroxydase and Sudan black B positivity in the cytoplasm reveal at the same time and in the same cells the myeloid way of differentiation, too

The characteristics of the subacute form of leukaemia, namely that the animals live for about 10–14 days after the appearance of the leukaemic blood picture and that it has kept its myeloid character even after serial transplantations, might promote further studies of the process of leukaemogenesis and testing of cytostatic agents with myelotropic potency. Studies in this respect are partly already under way, e.g., transplantability with cell free filtrate, ultrastructural and chromosomal investigations and the study of the reaction to the already known and to potential cytostatics. Attempts have been made to reveal and compare the behaviour of the 2 forms of leukaemia, the 'subacute' and 'ascitic' form to cytostatics.

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Acute Myelomonocytic Leukemia in a Patient with Hodgkin's Disease

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Abstract. A case report of a patient that developed acute myelomonocytic leukemia after Hodgkin's disease treated with total nodal irradiation is presented. The relationship between Hodgkin's disease and leukemia and in particular radiation leukemogenesis are discussed and it is concluded that treatment with ionizing radiation is the single most important etiological factor.

Key Words
Hodgkin's disease
Myelomonocytic leukemia
Radiation leukemogenesis

Occurrence of leukemia in patients with Hodgkin's disease is distinctly unusual. On the other hand, in patients with non-Hodgkin's lymphoma terminal leukemia occurs quite often [1, 2]. A sharp distinction has to be made between the so-called leukosarcomas, where the lymphoma itself becomes leukemic, and terminal appearance of leukemias such as acute myeloblastic (AML) or acute myelomonocytic (AMMoL) which do not have any obvious relationship to the lymphoma [3].

It is the purpose of this paper to present a patient with Hodgkin's disease who developed AMMoL approximately one year after total nodal irradiation and to discuss the relationship between the two diseases.

Case Report

The patient, a 29-year-old white female, was in good health until May 1971 when she first noted pain on cervical and [illegible] lymphadenopathy. On physical examination no abnormalities were found except for markedly enlarged right cervical and [illegible] lymph nodes. The diagnosis Hodgkin's disease was [illegible] histologically [illegible] (fig. 1).

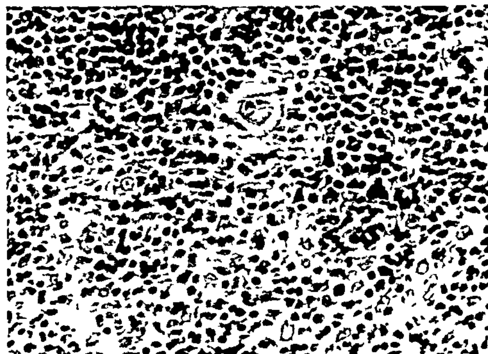


Fig 1 Lymphnode biopsy showing Hodgkin's disease mixed cellularity type HE, × 375

Complete blood counts sedimentation rate (Westergren) bone marrow cytology liver and kidney function tests were all normal

Staging including chest X rays with tomograms of the hilus and mediastinum lymphangiography X rays of the whole skeleton and exploratory laparotomy revealed the disease to be limited to the right cervical and supraclavicular region (stage IIA Rye classification)

The patient was treated elsewhere with 4 000 RTD from a ^{60}Co source to the neck the supra and infraclavicular region both axillae and the mediastinum Because of the histologic type of the Hodgkin's disease a prophylactic dose of 3 000 RTD was delivered in the fashion of an inverted Y to the thoracic and abdominal nodes Transient pancytopenia was noted 6 weeks after irradiation Afterwards the blood picture normalized completely

In April 1972 she started suffering from fatigue and easy bruising Laboratory studies disclosed the following values hemoglobin 6 7 g 100 ml hematocrit 20% white blood cells 2 200 mm³ platelets 48 000 mm³ The erythrocyte sedimentation rate was 67 mm in the first hour Bone marrow aspiration showed a marked shift to the left in myelopoiesis with some abnormal myeloblasts and promyelocytes No abnormalities were found in the other cell lines Preleukemia was suspected Treatment consisted of 4 transfusions of whole blood



Fig. 2. Bone marrow aspiration. Note the marked infiltration with myeloblasts and blast cells with monocytoid features. Giemsa [illegible]

In June 197[illegible] the [illegible] had fever, headache, sore throat and [illegible]. On admission to the University Medical Center Leiden in July 197[illegible] we found [illegible] petechiae and a periorbital hematoma on the right. A retinal hemorrhage was found [illegible] in the left eye. Besides the hemorrhagic [illegible] and marked [illegible] cervical lymphadenopathy and slight hepatomegaly were the only [illegible] findings on physical examination. Laboratory studies showed a sedimentation rate of [illegible] mm in the first hour. Hemoglobin [illegible] g/dl [illegible] white blood cell count [illegible] with the following differential: [illegible] myeloblasts, [illegible] promyelocytes, [illegible] metamyelocytes, [illegible] band neutrophils, [illegible] segmented [illegible] lymphocytes and [illegible] monocytes. Many of the blasts had monocytoid features and there were occasional Auer rods. The platelet count was [illegible]. A bone marrow aspiration [illegible] demonstrated a picture of leukemia [illegible] cells [illegible] therapy [illegible]

In the [illegible] between the courses she received [illegible] packed red cells. On discharge from the hospital she was in complete remission.

Discussion

[illegible] Hodgkin's disease and leukemia have been [illegible]

Table I Summary of reported cases of Hodgkin's disease and leukemia (group II)

Author	Year	Sex	Age years	Type of leukemia	Irradiation	Interval between irradiation and leukemia years
SKWORZOFF [15]	1903	F	8	acute myeloblastic	+ only spleen	0
DUBBERSTEIN [16]	1931	M	21	acute lymphatic	–	?
CRAVER [17]	1936	F	19	acute monocytic	+	?
WATSON [18]	1938	M	65	lymphatic	+	?
GILL and MACCALL [19]	1943	F	18	acute lymphatic	+	2
COHEN *et al* [20]	1958	F	26	acute stem cell	+	3½
RAZIS *et al* [9]	1959	F	27	monocytic	+	6
GREENBERG and COHEN [21]	1962	M	44	acute histiomonocytic	+	4½
LACHER and SUSSMAN [22]	1963	F	23	acute stem cell	+	1
SCHEERER *et al* [23]	1964	M	47	Reed Sternberg cell	+	½
CAVELLERO and BO [24]	1966	?	?	acute undifferentiated	+	?
HOLLARD *et al* [25]	1966	F	39	acute monoblastic	+	4
JOHNSON *et al* [26]	1966	F	39	chronic myelogenous	+	8
DURANT and TASSONI [27]	1967	M	37	acute erythroleukemic	+	10
EZDINLI *et al* [28]	1969	M	21	chronic myelogenous	+	9
EZDINLI *et al* [28]	1969	M	72	acute erythroleukemic	+	2
EZDINLI *et al* [28]	1969	M	33	acute myeloblastic	+	7
NEWMAN *et al* [29]	1970	M	28	acute myelomonocytic	+	18½
NEWMAN *et al* [29]	1970	M	26	acute myelomonocytic	+	1½
NEWMAN *et al* [29]	1970	M	38	acute myelomonocytic	+	5½
NEWMAN *et al* [29]	1970	M	68	acute myelomonocytic	+	4
REERINK *et al* [30]	1970	F	33	monocytic	+	6½
OSTA *et al* [31]	1970	M	60	acute myeloblastic	+	¼
STEINBERG *et al* [32]	1970	F	36	acute erythroleukemic	+	15
STEINBERG *et al* [32]	1970	F	30	acute myeloblastic	+	9
ROOZENDAAL *et al* [33]	1971	F	27	acute myelomonocytic	+	1½
ZWAAN *et al*	1972	F	20	acute myelomonocytic	+	1

(1) In some patients who died of chronic myelogenous leukemia (CML), Hodgkin's disease was found at *post mortem* examination [4, 5, 7]. Marked leukocytosis may occur in Hodgkin's disease, and it is possible that some of these patients did not have true CML [8, 9]. Chronic lymphocytic leukemia (CLL) precedes Hodgkin's disease more often [6, 7, 10–14]. In those instances it is likely that the patient suffered from two separate neoplastic diseases.

(2) In a larger group of patients, Hodgkin's disease is diagnosed and treated before the appearance of leukemia. In the world literature, we found a total of 27 cases (including our own patient). The frequency of the different types of leukemia in this group is summarized in table I [9, 15-33]. These leukemias are mostly acute myeloblastic or closely related. There is no sex predominance. All the patients were treated with ionizing radiation for their disease, except the cases described by SKWORZOFF [15] and DUBBERSTEIN [16]. Some also received systemic chemotherapy for their Hodgkin's disease. The interval between irradiation and appearance of leukemia in the entire group varied from 0 to 18.5 years, with a mean of 5 years. The relationship between Hodgkin's disease and leukemia can be as follows.

(a) A direct transition of the Hodgkin's disease into leukemia. This is unlikely to occur because of the different nature of the two diseases. The case of Reed Sterberg cell leukemia and possibly some cases of histiomonocytic – and pure monocytic (Schilling type) – leukemia may be exceptions.

(b) It has also been stated that patients with one neoplasm are prone to develop another one, which is not directly related [22, 25]. RAZIS *et al.* [9] studied a group of 1,102 patients with Hodgkin's disease and found 24 cases (2.2%) of other neoplasms of different types, from which carcinoma of the skin was most frequent.

(c) The most important etiological factor in the inductions of these leukemias is obviously ionizing radiation. Evidence for the relationship between irradiation and leukemia exists from studies of the Atomic Bomb Casualty Commission (ABCC) on Japanese survivors of the atomic bomb explosions [34-36], of patients with ankylosing spondylitis who received irradiation treatment to their spine [37, 38], of children who were exposed to diagnostic irradiation during pregnancy [39, 40].

From the studies of the ABCC, COURT BROWN and DOLL [38] and the patients with Hodgkin's disease treated with ionizing radiation the following can be said: at the end of 1962, 60 of the 15,554 patients studied by COURT BROWN and DOLL [38] had developed leukemia. The estimated excess mortality from leukemia was about 4 per 1,000 irradiated patients in an average follow-up period of 13 years. Three to 5 years after irradiation, the mortality from leukemia reached its maximum and then it declined. As in the group of patients with Hodgkin's disease, the leukemias in the series of COURT BROWN and DOLL [38] are usually acute myeloblastic or closely related.

From the studies of the ABCC it is known that there was an increased risk for the exposed people in Hiroshima and Nagasaki to develop leukemia if they were at 5 000 m or closer from the hypocenter of the explosions Within 12–18 months, there was an increase over the expected incidence of leukemia It reached a peak at about 7 years and remained significant for a total of 14 years

In the series of COURT-BROWN and DOLL and in Japanese A-bomb survivors, the incidence of induced leukemia at any given age is a constant multiple of the *natural incidence (and type)* of leukemia of the particular age group exposed [35, 36, 42] except for CLL [37, 43] The threshold dose that will induce leukemia is not known, but the incidence is estimated to be 1 3 cases per rad of whole body irradiation per million persons per year [41] There is suggestive evidence that even diagnostic X-rays at older age can be leukemogenic [44, 45]

It is well known from experimental and clinical studies that X-ray irradiation causes chromosomal aberrations and eventually activation of leukemogenic viruses [46–50] but it is not known if this is the mechanism of radiation leukemogenesis in man No statistical conclusions can be drawn from the series of RAZIS *et al* [9] and NEWMAN *et al* [29] about the incidence of acute leukemia in patients with X-ray irradiation for their Hodgkin's disease, but the fact that 2 out of 1,102 and 3 out of 1,500 patients, respectively, with Hodgkin's disease subsequently developed leukemia is high compared with the incidence of leukemia in a random population There is considerable evidence that cellular immunity is diminished in many patients with Hodgkin s disease [51] Treatment with ionizing radiation diminishes the cellular immunity further [52, 53]

Several of the patients we have summarized in table I had chemotherapy in addition to the radiation Chromosomal aberrations, diminished cellular immunity [54, 55] and a high incidence of AML had been reported in patients on aminopterin [56], melphalan [57, 58] and chlorambucil [59] So far 69 patients on chemotherapy for solid tumors have been reported to have developed AML or lymphoma and 22 cases of multiple myeloma terminated as AML after long term treatment with alkylating agents [60]

Even in Hodgkin s disease chemotherapy might be an additional leukemogenic factor A causal relationship between treatment with ionizing radiation and leukemia in patients with Hodgkin's disease is very likely, but other factors such as diminished immunologic capacity and treatment with cytostatic agents may have an important role as well

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Monoclonal Origin of Acute Transformation of Chronic Myelogenous Leukemia

Evidence from an Acquired Sex Chromosome Mosaic

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Abstract In a case of chronic myelogenous leukemia with an acquired sex chromosome mosaic (45, XO, Ph¹/46, XY, Ph¹), variant cells, developed after blast crisis, were evidenced to have been originated from a 46, XY, Ph¹+ clone because of the constant presence of a Y chromosome in them This strongly suggests that acute transformation has occurred selectively in a single clone

Key Words
Acute transformation of myelogenous leukemia
Cell clone in leukemia
Chromosome mosaic
Karyotype in leukemia

Cytogenetic studies have provided the evidence of clonal proliferation in human malignant disease [3] and further suggested that tumors arise frequently from a single cell with chromosomal aberrations [1, 11] The question whether a tumor is unicellular or multicellular in origin might be clearly answered by chromosome studies of cases with a sex chromosome mosaic, but it is a rare event

The present paper reports the monoclonal occurrence of acute transformation in chronic myelogenous leukemia with an acquired sex chromosome mosaic

Materials and Methods

Chromosome preparations of bone marrow cells were made by a direct method with a modified technique of TJIO and WHANG [8] The heparinized marrow aspirates (0 5-1 0 ml) were immediately incubated in 2 ml of TC 199 culture medium containing a few drops of 50 μg ml colchicine solution, for 30 min at room temper

[illegible]. This was followed by hypotonic treatment with 0.6% sodium citrate and then by the standard fixation and the flame drying technique. The peripheral leukocyte culture was set up according to the method of Moorhead *et al.* [6]. After staining with Giemsa, suitable metaphases were counted, photographed and analyzed in each sample.

Case History

I.M., a 27-year-old, phenotypically normal male was admitted because of splenomegaly and leukocytosis in December 1970. Hematologic examinations showed hemoglobin 13.0 g/[illegible], platelets 314 × 10³/mm³, white cell count 118 × 10³/mm³, myeloblasts 4%, promyelocytes 3%, myelocytes 6%, metamyelocytes 4%, band cells 17%, polymorphs 39%, eosinophils 6%, basophils 8%, monocytes 1%, lymphocytes [illegible]. A bone marrow aspirate showed a marked granulocytic hyperplasia: myeloblasts 4.6%, promyelocytes 21.6%, myelocytes 10%, metamyelocytes 11.6%, band cells [illegible]%, polymorphs 12.6%, and erythroblasts 5.2%. The diagnosis of chronic myelogenous leukemia was made on the above findings together with a low NAP score ([illegible]). Treatment with busulfan (2 mg daily) induced rapid improvement in clinical and hematological conditions, followed by a 4-month remission. In July 1971, however, a bone marrow specimen revealed moderate increase in myeloblasts (25.2%) and the diagnosis of acute transformation was made. Two months later, the patient complained of fever, back pain and malaise, and circulating blast count increased rapidly with anemia and thrombocytopenia. Combination treatment with prednisolone, 6-mercaptopurine, methotrexate and blood transfusion gave a 4-month remission, during which a severe pulmonary infection had occurred and leukocyte count dropped below [illegible]/mm³. In February 1972, however, a bone marrow aspirate showed again an increase in blasts and combination therapy with prednisolone, 6-mercaptopurine, methotrexate and vincristine induced a complete remission. About 4 months later, the third relapse occurred with predominant myeloblasts in marrow and blood and the patient died from a gastric hemorrhage at the beginning of August 1972, 13 months after the diagnosis of acute transformation.

Cytogenetic Findings

Chromosome studies of bone marrow cells were repeated many times. In the chronic stage, two cell lines with 45,XO,Ph¹ and 46,XY,Ph¹ karyotypes were present at the ratio of about 1:1 both in marrow and [illegible] blood culture (Fig. 1). In the karyotype of peripheral lymphocytes cultured for 72 h with PHA at a remission showed 46,XY only. During 4 months after acute transformation there were no karyotypic changes in [illegible] but the ratio of 45,XO,Ph¹ cells increased gradually. However, the [illegible] of April 1972 [illegible]

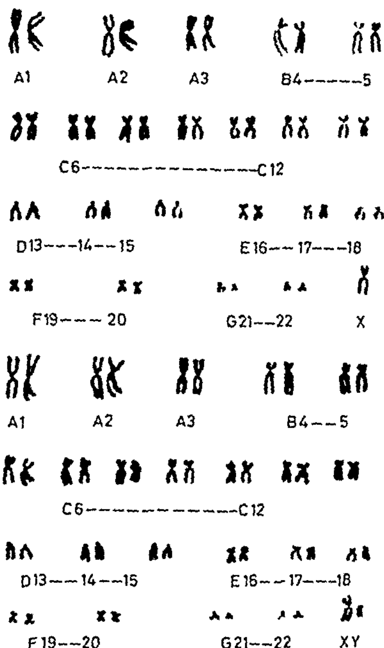


Fig. 1. Karyotypes of cell lines with 45 (top) and 46 (bottom) chromosomes in the chronic stage. The former lacked the Y chromosome (top) but both lines contained the Ph¹ chromosome.

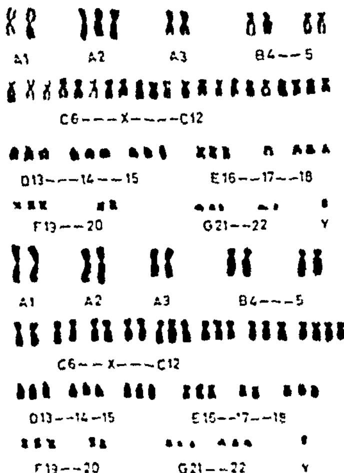

A1
A2
A3
B4——5
C6———X————C12
D13———14——15
E16——17——18
F19——20
G21——22
Y
A1
A2
A3
B4———5
C6——X———C12
D13——14—15
E15——17——18
F19——20
G21——22
Y

Fig. 2 [illegible]

[illegible]

Discussion

The developement of chronic myelogenous leukemia in individuals with a sex chromosome mosaic has been reported quite rarely [5, 9]. In a reported case with an acquired XO/XY mosaic, cytogenetic studies after acute transformation were not described [10]. The mosaic of bone marrow cells in the present case was considered to be an acquired one which might probably be produced in the leukemic process, because peripheral lymphocytes showed a normal male karyotype. The chromosome constitutions of polyploid cells with 90 and 92 chromosomes were the exact doubling of 45, XO, Ph¹ and 46, XY, Ph¹ karyotypes, respectively, and it is suggested that anaphase lagging of Y chromosome had happened in a 46, XY, Ph¹ stem cell, resulting in the production of two kinds of stem cells with and without a Y chromosome.

Monoclonal origin of chronic myelogenous leukemia has been strongly supported by cytogenetic [5, 11] and isoenzymatic studies [4] and it is suggested that karyotype evolutions are frequently, if not all, the cytogenetic basis of acute transformation of chronic myelogenous leukemia [2, 7]. In the present case, the greatest interest is whether acute transformation might occur in a single clone (45, XO, Ph¹ or 46, XY, Ph¹ cells) or in both. Detailed analysis of variant cells developed after blast crisis constantly revealed a Y chromosome. This is a clear cytogenetic evidence that these variants were originated from a single clone with 46, XY, Ph¹ karyotype and strongly suggests that acute transformation has occurred selectively in a single clone.

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Influence d'acides gras saturés et non saturés sur la pression de filtration de plasma riche en plaquettes. Corrélation avec l'agrégation plaquettaire[1]

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Abstract The influence of the salts of saturated and non-saturated fatty acids from C8 to C22 on the aggregation of human platelets is compared within the framework of 2 different techniques the test of filtration pressure, in correlation with the classical photometric test of aggregation

Key Words
Fatty acids
Filtration pressure
Platelet aggregation

Thus, with the technique of filtration pressure of platelet rich plasma, one finds an increase in filtration pressure as a function of the number of carbon molecules in the chain used The presence of a double bond generally results in a decrease in filtration pressure.

In the classical photometric test the fatty acids induce a progressive and irreversible deviation curve, demonstrating both the aggregation of platelets and the probable bonding of the fatty acids with plasmatic proteins. The presence and intensity of platelet aggregation are related to the molecular weight of the chain of fatty acids under consideration and inhibited by the presence of double bonds.

From the similarity of the results obtained with the 2 techniques, one might surmise that the filtration pressure test is a new approach to platelet aggregation

Les travaux concernant l'action des sels d'acides gras vis-à-vis de l'agrégation plaquettaire restent peu nombreux depuis les premières publications de CONNOR et POOLE [2] en 1961, qui montraient l'activité agrégante et thrombogène des sels d'acides gras saturés à longue chaine Le mécanisme d'action de ces agents reste discuté. Le rôle de l'ADP

[1] Travail réalisé avec l'aide de la DRME (Section Biologie), Contrat n° 72.34.238 00 480 75.01

Tableau I. Sels sodiques d'acides gras utilisés (concentrations équimolaires à 4 mM, pH 8)

Nom	Nombre de C	Nombre de doubles liaisons
Chaînes saturées		
Caprylique	8	pas de doubles liaisons
Caprique	10	
Laurique	12	
Myristique	14	
Palmitique	16	
Stéarique	18	
Béhénique	22	
Chaînes insaturées		
Oléique	18	1
Linoléique	18	2
Linolénique	18	3
Érucique	22	1

médiateur a été suggéré par HASLAM [4] qui a montré l'existence d'une inhibition de l'agrégation en présence d'adénosine. Cependant, d'autres auteurs, et en particulier DAWBER et ROBERTS [3], incriminent l'intervention de modifications physicochimiques intervenant au niveau du potentiel zêta, qui serait diminué en présence des acides gras à longue chaîne et augmenté en présence des acides gras fortement insaturés comme l'acide linoléique.

Ce travail tente d'apporter de nouveaux arguments dans la discussion en étudiant la corrélation éventuelle entre l'agrégation des plaquettes induites par les acides gras et le test de la pression de filtration.

Matériel et méthodes

Les sels d'acides gras [illegible] ont été préparés selon les données de [illegible] et [illegible] sous forme de suspension [illegible] à [illegible] par la soude à pH 8. La pureté des acides gras [illegible]. Les [illegible] de sels d'acides gras à longue chaîne [illegible]. Ce [illegible]

* [illegible] France

Tableau II Influence de sels d acides gras sur la pression de PRP (mm Hg)

	Témoin diluant	(a) Acides gras saturées							(b) Acides gras insaturées			
		C8	C10	C12	C14	C16	C18	C22	C18 1	C18 2	C18 3	C22 1
8 ml PRP +1 ml AG	6 ±4	6 5±4	7 ±4	6 ±4	5 ±4	20±8	16±5	23±9	7 ±4	6 ±4	7,5±4	9±5
8 ml PRP +2 ml AG	5 ±4	6 ±4,5	7,5±4 5	7,5±4	4,5±3,5	45±19	27±8	68±14	7,5±4	6,5±4	7,5±4,5	12±6
8 ml PRP +3 ml AG	6,5±4,5	5,5±4,5	5 ±4,5	8 ±4,5	6 ±4,5	51±21	65±12	85±17	9 ±5	5 ±4,5	6,5±4	14±8
8 ml PRP +4 ml AG	5 ±4,5	5 ±4	5 ±4	10 ±5	7 ±4	91±30	74±14	125±29	20 ±6	5,5±4	7,5±4	24±11
8 ml PRP +5 ml AG	7,5±4	6,5±4	6 ±4,5	10 ±5	7,5±4,5	87±32	103±26	135±35	12 ±5	5 ±4	8 ±4,5	31±14
Nombre de mesures	9	4	6	5	3	10	6	3	4	3	5	6

Tableau III. Influence de sels d'acide gras sur l'agrégation plaquettaire. Étude de la chute de DO après 3 et 5 min d'agitation (concentration en AG = 1,31 mM)

	Chute DO après 3 min, %	Chute DO après 5 min, %	Nombre de mesures
(a) Acides gras saturés			
Témoin d'essai	0	0	10
C8 à C12	0	0	20
C14	5,0 ± 1,1	5,2 ± 1	5
C16	25,2 ± 0,4	34,4 ± 9,4	5
C18	23,9 ± 6	45,5 ± 4	6
C22	42,8 ± 3,4	74,2 ± 10,9	6
(b) Acides gras insaturés à longue chaîne			
Témoin d'essai	0	0	10
C18:1	3,1 ± 2	6,1 ± 1	5
C18:2	0	0	3
C18:3	0	0	3
C22:1	56,5 ± 9,2	62,2 ± 5,4	6

l'ensemble des sels résolus et à la solution d'azote. Les suspensions ont été ramenées rapidement à 37 °C avant les tests.

La pression de filtration a été déterminée à l'aide de l'appareil décrit initialement par Swank et Davis [13] et avec une standardisation [illegible] des mesures [11, 12]. Les filtres utilisés ont toujours été des filtres métalliques à pores cubiques de 20 µm. Les sels d'acides gras ont alors été étudiés par addition de volumes constants (1,5 ml) à 8 ml de *PRP, ce qui faisait varier la concentration finale* en sels de 0,41 à 1,31 mM. Les mesures ont été réalisées à 25 °C, environ 2 min après l'adjonction de l'acide gras testé.

Les essais d'agrégation ont été effectués sur un agrégomètre du type original de Mustard [9] à la température de 37 °C avec agitation de 1100 t/min et couplé à un enregistreur [illegible] de 2 cm [illegible].

Le plasma riche en plaquettes (PRP) test était issu de sang recueilli sur citrate de sodium. Il est obtenu par centrifugation modérée à [illegible] de plaquettes [illegible] de 250 000 ± 25 000. Il est [illegible] dans les proportions de 0,5 ml pour 0,15 ml de [illegible] d'acides gras, dont les concentrations finales ont varié de 0,41 à 1,31 mM. Une étude parallèle a été réalisée avec le plasma déplaquetté (PPP).

Résultats

Pression de filtration. Les résultats obtenus sur la pression de filtration montrent que parmi les acides gras saturés (tableau IIIa) les acides à

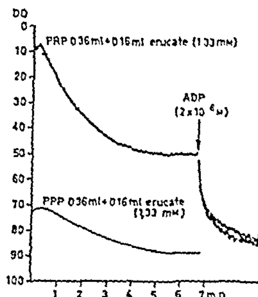


Fig 1 Action de l addition d ADP sur la taille des agrégats préalablement induits par erucate (37 °C 1000 tours min)

courtes chaines (jusqu à C14) n'induisent pas de modifications importantes de la pression de filtration alors que pour les longues chaines de C16 à C22, on peut noter une nette augmentation de la pression de filtration proportionnelle à la concentration de l acide gras utilisé De plus la présence d une double liaison induit une diminution importante de la pression de filtration comparée à une chaine de même longueur saturée (tableau IIb)

Agrégation photométrique L'étude révele qu'avec nos conditions expérimentales, seuls les sels d acides gras à longue chaine (supérieur à *C14*) sont capables d induire le phénomène d agrégation et que les courbes d agrégation alors obtenues présentent différents caractères particuliers qui ne permettent pas de les superposer à celles des autres agents agrégants classiques D apparition progressive et irréversible, elles semblent correspondre à l'enregistrement photométrique d un double phénomène l un plaquettaire se traduisant par une diminution de la densité optique en relation avec l agrégation des plaquettes, l autre plasmatique lié à l éclaircissement du plasma lui même rendu opaque après addition de la suspension du sel d acide gras étudié De plus la vitesse et l intensité de l agrégation semblent inversement proportionnels à la longueur de la chaine utilisée

Etant donné que la forme des courbes enregistrées semble être grandement influencée par ce phénomène plasmatique et ces constatations nous ont conduit à introduire un facteur de correction établi d'après la courbe obtenue par les sels d'acides gras avec le PPP pour le calcul de la diminution de la densité optique du PRP traduisant l'agrégation plaquettaire proprement dite.

Cette agrégation, exprimée d'après le pourcentage corrigé de la diminution de densité optique à la 3e et à la 5e min est en liaison étroite avec la longueur de la chaîne de l'acide gras et avec son degré de saturation (tableau III) : nulle jusqu'à C12, l'intensité de l'agrégation augmente de C14 à C22 pour les acides gras saturés tandis que la présence d'une double liaison atténue le phénomène et que deux ou trois doubles liaisons l'annulent. Dans ce système l'érucate possède une activité intermédiaire entre le stéarate et le béhénate.

Discussion

Les résultats rapportés ci-dessus permettent de conclure en une bonne corrélation entre la technique photométrique et la pression de filtration pour l'appréciation de l'agrégation plaquettaire. Cependant, pour les sels d'acides gras insaturés, avec une double liaison et en particulier pour l'érucate, il faut noter une discordance de réponses : en effet, cet agent induit une chute importante de la densité optique de l'agrégomètre alors qu'il n'entraîne qu'une relativement faible augmentation de la pression de filtration. Une telle différence ne semble explicable ni par la cinétique de la réaction, ni par une éventuelle réversibilité de l'agrégation.

Par contre, il est possible d'envisager l'intervention de la taille des agrégats comme pourrait le suggérer l'augmentation photométrique de cette [illegible] après addition d'ADP [9] à des plaquettes préalablement agrégées par l'érucate (fig. 1) ou un problème de rétention des agrégats au niveau du filtre comme nous avons déjà pu le constater avec certains produits tel que le polybrène [12].

Enfin, parmi les hypothèses pouvant expliquer nos résultats, il faut noter que seuls les sels d'acides gras saturés à longue chaîne sont capables d'activer le facteur contact XII → XI et d'induire chez l'animal l'apparition d'un thrombus dans un segment de veine jugulaire ligaturé [11] ; l'intervention éventuelle du facteur Hageman dans l'adhésion des plaquet-

tes avancée dès 1962 par JÜRGENS [8] ne semble pas avoir été retenue par les travaux plus recents, et en particulier ceux de BARTH *et al* [1] l'interaction des paquettes et du système contact parait cependant étroite [14] On peut supposer également que l'activation des facteurs XII et XI, adsorbés au niveau de la membrane plaquettaire [6, 7] conduisent à la formation de thrombine dont l'action agrégante pourrait expliquer la différence de comportement des agrégats

Résumé

L'influence des sels d'acides gras saturés et insaturés de C8 à C22 sur l agrégation des plaquettes humaines est comparée dans le cadre de deux techniques differentes le test de la pression de filtration, en corrélation avec le test classique d'agrégation photométrique C'est ainsi qu'avec la technique de la pression de filtration de plasma riche en plaquettes on trouve une augmentation de celle-ci en fonction du nombre de molécules de carbone de la chaine utilisée La présence d'une double liaison induit généralement une diminution de la pression de filtration Dans le test classique photométrique, les acides gras induisent une courbe de déviation progressive et irréversible, témoin à la fois de l'agrégation des plaquettes et de la conjugaison vraisemblable de l'acide gras avec les protéines plasmatiques L'existence et l'intensité de l'agrégation plaquettaire sont liées au poids moléculaire de la chaîne d'acides gras considérée et inhibée par la présence de doubles liaisons La similitude des résultats obtenus avec les deux techniques permet de penser que le test de la pression de filtration est une approche nouvelle de l agrégation des plaquettes

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Fetal Factor VIII and IX Levels in Early Pregnancy and their Significance in Prenatal Diagnosis

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Abstract Following the failure to discover significant factor VIII and IX levels in a 14-week male fetus, from a pregnancy terminated because the mother was a known carrier of Christmas disease, these factors were measured in 10 other apparently normal fetuses In no case was the level above 1% Factors VIII and IX therefore, do not appear to be synthesised in early pregnancy, so it is not possible to differentiate normal from haemophilia and Christmas disease males by present techniques during the early stages of pregnancy when termination would be acceptable

Key Words
Christmas disease
Factor VIII and IX in fetal life
Haemophilia
Pregnancy
Prenatal diagnosis

In the last few years, the development of techniques of antenatal identification of the sex of a fetus has meant that genetic counselling of families, in respect of the sex linked disorders, has been able to assume a more positive role Formerly a haemophilic male could only be advised that all his sons would be normal and all his daughters would be carriers, a known carrier of Duchenne muscular dystrophy could only be advised that each of her sons would have a 50 percent chance of developing the disorder, and that each of her daughters would have a 50 percent chance of being a carrier Today, patients with sex linked disorders and carrier women, known either from their family history or (an increasing proportion) from carrier detection tests, can be helped in the planning of their families by the offer of selective termination according to fetal sex

In the case of haemophilia and Christmas disease, it is possible to identify a proportion of female carriers by detecting reduced levels of factors VIII and IX, respectively, in their blood One is able to offer these

women selective abortion of male fetuses, by performing amniocentesis and sexing the fetus from desquamated cells floating in the liquor amnii. Termination of pregnancies where the fetus is male will ensure that the mother has only female children, outwardly normal although each has a 50 percent risk of being a carrier. Unfortunately, however, 50% of the male fetuses of the terminated pregnancies will be normal. Were it possible to estimate directly fetal factor VIII and IX levels *in utero*, to differentiate affected and non affected males, the offer of termination of such pregnancies could be made on a much more precise basis. It is not, however, known at what gestational age synthesis of these factors to a detectable level commences. The problem is illustrated by the following case.

Case Report

Mrs. E. R. was referred for prenatal sexing during her fourth pregnancy, because she was a known carrier of Christmas disease (her factor IX level [illegible]%), both her father and paternal uncle having suffered from the disease. One of her previous pregnancies had ended in a miscarriage, but the other two had both produced male children both suffering from Christmas disease. The first child was alive, aged [illegible] years, with a history of a common sequence of haemarthroses and other episodes, quite severely affected. The second child had died at [illegible] years, and had been mentally retarded, this being attributed to early intracranial haemorrhage. The patient dreaded a further affected boy. Her husband informed us that he had grave doubts of his own ability to stand the worry. Both parents expressed the wish to have a male fetus aborted.

Transabdominal amniocentesis was, therefore, performed during the 16th week of pregnancy, and [illegible] ml of clear liquor amnii removed without difficulty. The fetal cells were stained for Barr bodies with [illegible] [illegible], and no positive ones were found in 100 cells examined. Similarly, staining with 0.5% quinacrine hydrochloride to detect the Y chromosome by fluorescence gave [illegible] positive in 200 cells examined. The fetus was, therefore, sexed as male. The result was later confirmed by chromosome analysis of the cultured cells. On the result of the sexing the pregnancy was terminated by abdominal hysterotomy. A specimen of fetal blood was obtained immediately the fetus was delivered and the factor VIII and IX levels were estimated. Both levels were less than 1%. On reviewing the literature, although normal factor VIII and IX levels in infancy are known, no information was available on the presence of levels in fetal life [illegible] whether or not factor IX in the present case was normal.

Procedure

Factor VIII and IX levels were, therefore, estimated in fetuses from [illegible] pregnancies terminated for other indications [illegible] where there was no suggestion of any form of blood clotting disorder in the families concerned.

Table I

Gestation length (from LMP), weeks	Factor VIII level, %	Factor IX level, %
11	<1	<1
12	<1	<1
13	<1	<1
14	<1	<1
14	<1	<1
14	<1	<1
14	<1	<1
14[1]	<1	<1
15	<1	<1
19	<1	<1
22	<1	<1

[1] The mother is a known carrier of Christmas disease

The fetal blood samples were obtained after delivery of the fetus, by either direct aspiration of an umbilical vein, or division of the cord and drainage. The blood was collected in a bottle containing 3.8% sodium citrate, so that a mixture of 1:9 citrate to blood was formed. Factor VIII was measured by a two-stage method based on that of Biggs and Macfarlane [1962].

The test plasma and a normal standard were prepared by absorption with aluminium hydroxide and diluted with veronal buffer on the day of test and serial concentrations were made. The time taken for each preparation to induce coagulation in a sample of haemophiliac plasma was then measured, and the level of factor VIII in the test plasma calculated from this.

Factor IX was measured by a one stage method based on the activated partial thromboplastin test (APTT). The method is similar in principle to that described for factor VIII, except that the degree of correction of coagulation given by the test plasma when added to severe factor IX deficient plasma, compared to that given by normal plasma, is used to calculate the percentage activity.

Results

The results are set out in table I according to the fetal age. The fetuses ranged from 11 to 22 weeks, and in each of these the factor VIII level was less than 1%. Similarly, the factor IX level was also less than 1%, though there was a suggestion in the oldest fetus (22 weeks) that the fac-

tor IX level may be beginning to rise. The fetus from the original carrier patient is not in any way distinguished from the other normal fetuses of this age.

These results are perhaps not surprising, in view of the known irregularity of blood clotting in the early fetus. They are, however, somewhat disappointing, for with the current rapid advances in obstetric techniques, it will probably be possible, in a few years' time, to safely obtain a fetal blood sample in early pregnancy. One of the fields in which this would be of most relevance is in the inherited blood clotting disorders. Unfortunately, the present results imply that the relevant serum factor levels in normal fetuses are too low for differentiation between normal and affected by present assay methods, certainly within the gestation limits in which pregnancy termination on genetic grounds would be acceptable.

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ROSEMARY BIGGS (ed) **Human Blood Coagulation, Haemostasis and Thrombosis.** Blackwell, Oxford

The new edition of the 'Human Blood Coagulation and Its Disorders', originally written by ROSEMARY BIGGS and R G MCFARLANE shows an important change in its conception The three previous editions were written by two authors having a large part of their great experience in common The book was accordingly homogeneous and the theories based first on the experimental work of the Oxford team and less on literature

This character has been necessarily lost due to the new policy of the editor ROSEMARY BIGGS This does not mean that the quality is less than for the previous editions In some ways it is an improvement The tremendous development of knowledge in this field can much better be taken into consideration when the particular chapters are written by specialists This provides more details for many chapters and has allowed the introduction of entirely new aspects, particularly the chapters reserved to the clinic of blood coagulation, the fibrinolysis and especially the thrombolytic therapy, the defibrination syndrome, the anticoagulant therapy

The new edition may be for the student or unspecialized reader less easy to read and to assimilate because he is confronted with different concepts and presentations This is only a minor inconvenient in comparison to the value and extension of the information 'Human Blood Coagulation, Haemostasis and Thrombosis' can be recommended as one of the best books at the moment for students, specialists and certainly for clinicians F DUCKERT, *Basel*

M RORTH **Hemoglobin Interactions and Red Cell Metabolism.** Series Haemat., vol V/1 Munksgaard, Copenhagen 1972 104 pp, dKr 60 –

The monograph on hemoglobin and red cell function is divided in 3 parts In the first section, the stereochemistry of hemoglobin, the binding of ligands other than oxygen to hemoglobin the transport of oxygen and carbon dioxide in whole blood and the methods for determination of the oxyhemoglobin dissociation curve are reviewed and discussed Part 2 deals with the metabolic regulation of glycolysis, the function and regulation of 2,3-diphosphoglycerate, and methods for the determination of glycolytic intermediates. In part 3, changes in the oxyhemoglobin dissociation curve under different physiological and pathological conditions such as hypoxia acid base disturbances, and blood storage are discussed

The experimental work carried out by the autor himself at the Department of Clinical Chemistry, Ringhospitalet, Copenhagen is included in the review A list of more than 200 references and a subject index conclude the monograph which constitutes a valuable source of information for laboratory investigators and clinical hematologists interested in hemoglobin and red cell research H R MARTI, *Aarau*

P L MOLLISON **Blood Transfusion in Clinical Medicine, 5th ed** Blackwell Oxford 830 pp £ 8 00

There is certainly no need to comment on the basic concept of this well known monograph. Indeed MOLLISON's book represents one of the standard works, if not

the standard work on transfusion medicine. We are, therefore, greatly indebted to the author for preparing periodically new revised editions. The present 5th edition has again been brought up-to-date in the usual skillful and competent manner. Special attention has been given to important recent developments, such as the prophylaxis of the haemolytic disease of the newborn due to Rh incompatibility. All practical aspects of this subject are treated exhaustively including, for information about the amount of anti-D immunoglobulin required for a successful suppression of sensitization. Since the publication of the last edition of the book, a number of new subjects have become of considerable interest in blood transfusion. They are all dealt with in the 5th edition and include topics such as the role of anti-IgA antibodies in inducing anaphylactoid transfusion reactions as well as the implications of the HL-A system in blood transfusion (especially platelet transfusion), bone marrow transplantation, etc. In addition, experience with the use of cryoprecipitate in the treatment of haemophilia and with a new, more alkaline stabilizer for stored blood (CPD) is reported. Undoubtedly, a discovery with the greatest impact on blood transfusion is the recent establishment of the relationship of hepatitis B antigen (Australia antigen) to posttransfusion hepatitis. For this subject again, the author presents a wise and critical selection of information. In spite of the integration of these new chapters into the 5th edition, the total size of the book does not exceed that of the previous edition. This has been achieved by dropping some subjects with less direct implications for blood transfusion such as, e.g. serum group systems other than immunoglobulin groups. The second edition of Mollison's monograph has been awaited with eagerness by everybody involved in blood transfusion. It belongs in the library of every blood bank and hospital, as well as in every immunohaematological laboratory.

R. BÜTLER, *Bern*

Ontogeny of Acquired Immunity, A Ciba Foundation Symposium. Elsevier Excerpta Medica North-Holland, Amsterdam-London-New York 1972. DF 31.- (approx. $ 12.-)

The present volume offers a full report on the Ciba Foundation Symposium on the ontogeny of the immune response. To know the normal development of immunological competence and its regulation should provide insights into the immune deficiency syndromes due to errors of development.

As is fitting to the topic, the volume begins with the theoretical base of all immunological development. N. K. Jerne presents his expansion of the clonal selection theory. The first group of papers treats the origin and development of lymphocyte populations (J. J. T. Owen) and the ontogeny of complement components and lysozyme (M. Adinolfi). A. M. Silverstein then discusses a fascinating topic, the modulation of infectious diseases during the immunological maturation of the fetus. The next group of papers deals with the physiological non-reactivity of the immune system, whether due to allotype suppression (L. Herzenberg), to blocking antibodies and complexes (K. E. and I. Hellström), or due to the specific situation of the fetus in the maternal uterus (P. B. [illegible] and A. E. Beer). The [illegible] of the complement (P. J. Lachmann) and of the [illegible] (P. S. [illegible]) [illegible] by the [illegible] bone marrow [illegible] (D. W. van Bek[illegible]

kum and K. A. Dicke) or by fetal thymus grafts (H. C. M. Kay). Each paper is followed by a well-edited discussion which is sometimes as interesting to read as the papers themselves. All reports are concise, bringing original data pertinent to latest trends in immunological thinking. The present volume can be highly recommended to everybody with an interest in immunology. T. L. Vischer, Ge

G. Seifert, Hrg.: Aktuelle Probleme der Kinderpathologie. Hauptthema der 55. Verhandlungen der Deutschen Gesellschaft für Pathologie 1971. 1972. XII + 565 pp., 311 fig., 101 tab., DM 110.–

Die 55. Verhandlung der Deutschen Gesellschaft für Pathologie 1971 war im Hauptthema aktuellen Problemen der Kinderpathologie gewidmet. Dabei wurden mehrere Themenkreise behandelt. Dem 1. Thema, Atemnotsyndrom, sind 7 Vorträge gewidmet, die eine Fülle von physiologischen und klinischen Daten neben den eigentlichen histopathologischen Befunden präsentieren. Auch über die Korrelation klinischer, röntgenologischer und histologischer Befunde wird berichtet. Das 2. Thema, Viruspneumonie, wurde von H. Münterfering ausführlich behandelt, wobei sehr instruktive mikroskopische Bilder präsentiert werden. Insgesamt 9 Vorträge befassen sich mit kindlichen Endokrinopathien, wiederum mit einem klinischen Einleitungsreferat. Im weiteren kommen besonders Nebennieren- und Hodenpathologie zur Sprache. Es folgen Vorträge über Probleme des kindlichen Immunsystems, eingeleitet mit einer Besprechung des lympho-retikulären Systems und der Klinik kindlicher Immunopathien sowie Vorträge zur Pathogenese frühkindlicher Tumoren und Leukämien. Im Eintrittsreferat werden auch die therapiebedingten Organveränderungen aufgeführt. Mit Verbrauchskoagulopathien im Kindesalter befassen sich 2 Beiträge. Nicht weniger als 13 sind den angeborenen Stoffwechselkrankheiten gewidmet. Neben Fructose-Intoleranz und Galaktosämie werden u.a. Gangliosidosen, Glycogenosen, Mucopolysaccharidosen und Mucolipidosen behandelt. Ein weites Feld umfasst der letzte Themenkreis, freie Vorträge. Zum Schluss wird in einem Rundtischgespräch der unerwartete plötzliche Tod im Kindesalter diskutiert und von vielen Seiten beleuchtet.

Der vorliegende Band befasst sich mit zahlreichen, besonders aktuellen Themen der kindlichen Pathologie, wobei neben lichtmikroskopischen und elektronenmikroskopischen Befunden auch die klinischen, pathogenetischen, enzymatischen und Laboraspekte berücksichtigt werden. Er ist deshalb nicht nur für den Pathologen, sondern besonders auch für den Kliniker von grossem Interesse. Besonders hervorzuheben sind die durchwegs ausgezeichneten Abbildungen. M. Vest, *Basel*

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Oxymetholone Treatment in Hypoproliferative Anaemia[1]

I. Frequency of Response

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Abstract. 45 patients with hypoproliferative or aplastic anaemia were treated with oxymetholone, an anabolic androgenic steroid. Minimum treatment time accepted for entrance into the study was 3 months. Therapeutic effect was assessed by effect on bone marrow cellularity, haemoglobin concentration and granulocyte and platelet counts. Patients with a [illegible] marrow responded best. Of 33 cases, a partial remission was seen in 8 and a complete remission in 5. A complete remission was only seen in patients who did not have pancytopenia. Of the 7 patients with myelofibrosis, 4 had thrombocytopenia at the outset. Improvement after treatment was seen in all 4. The only serious side effect that necessitated withdrawal of treatment was jaundice.

Key Words
Anabolic androgenic steroid
Anaemia therapy
Aregenerative anaemia
Bone marrow cellularity
Oxymetholone

Shahidi and Diamond [14] and Gardner and Pringle [6] first reported improvement of aregenerative and chronic refractory anaemia after treatment with testosterone in 1961. Later studies revealed that improvement occurred in only a few adults [5, 9, 13, 16]. In 1964, Sanchez Medal *et al.* [11] reported remission in 75% of cases of various types of chronic refractory anaemia after treatment with a new anabolic steroid, oxymetholone. Several more recent reports [2, 7, 12, 15] have [illegible] these findings.

The aim of this study was to determine the type of hypoproliferative anaemia that best responds to a standard, high-dose treatment [illegible]

[1] [illegible]

Table I Total material diagnosis age, sex and duration of anaemia

	Diagnosis	Number of evaluated patients	Sex female	Sex male	Age years range	Duration of anaemia months median value	Duration of anaemia months range
oup I	*Aregenerative anaemia*						
	Hypocellular with pancytopenia	6	2	4	15–61	3 5	1–24
	Hypocellular with neutropenia or thrombocytopenia	12	8	4	6–75	4	1–48
	Normocellular	4	2	2	67–75	24	2–60
	Hypercellular	6	2	4	41–78	6	1–12
oup II	Erythroblastopenia						
	Congenital	3		3	6–16	since birth	
	Acquired	3	3		10–46		6–>30 years
oup III	Myelofibrosis	7	2	5	39–75	8	4–72

gime Because of the small number of cases expected to be seen at just one centre, a multi-centre trial was set up The therapeutic effect up to the time of the first remission in those patients seen in the first 18 months of the trial will here be reported

Material

45 patients with hypoproliferative aregenerative or chronic refractory anaemia entered the investigation All new patients diagnosed during the period of the study as well as those patients already under treatment when the trial began were included Four patients died before 3 months treatment had been completed and were, therefore withdrawn from the study This was the minimum time selected for treatment, when no earlier response was seen 19 of the 41 remaining patients were female and 22 male Ages ranged from 6 to 78 years and the mean duration of anaemia from 4 to 24 months

Patients were divided into 3 main groups (table I) Group I included 28 patients with hypoproliferative or aregenerative anaemia Hypoproliferative anaemia was defined as a refractory anaemia which was not due to any other disease process and in which haemolysis played no major role Group I was further subdivided into 3 subgroups based on their bone marrow cellularity as judged in sections of bone marrow The first subgroup included those patients with a hypocellular marrow the second those with a normocellular and the third those having a hypercellular marrow In addition the patients with hypocellular marrows were further subdivided

[illegible] oxymetholone treatment: duration (months) and doses (mg/day) in relation to the therapeutic response and to serious side-effects

[illegible] response — 250 — 150 — 0 — A

Serious side-effects

no — 250 — 125 — 75 — 0 — 75 — B

yes — 250 — 0 0 — 75 — 0 — 75 — C

no — 250 — 125 — 75 — 50 — 0 — 50 — D

yes — 250 — 125 — 0 0 — 75 — 50 — 0 — 50 — E

1 2 3 4 5 6 7 months

[illegible]

into those with pancytopenia, and those having a granulocytopenia or a thrombocytopenia. Six patients with congenital or acquired erythroblastopenia made up group II and 7 patients with proven myelofibrosis group III

Methods

Oxymetholone (Anasteron®, 50 mg Astra Syntex, Sweden) in a synthetic, anabolic steroid that may be orally administered Dosage and duration of treatment in this trial are shown in table II For the purposes of this study a complete remission was defined as complete normalisation of haemoglobin concentration granulocyte count and platelet count A partial remission was defined as a rise in haemoglobin concentration of at least 3 g/100 ml during a period free from blood transfusions, or an increase in platelet count of 50 000 cells/μl No isolated effect on granulocytes was seen in this study

Results

Effect on haemoglobin concentration (table III) Of 18 patients in group I with hypocellular marrows, 6 had an almost aplastic marrow with peripheral blood pancytopenia Four of these 6 improved as indicated by a rise in mean haemoglobin concentration from 6 8 to 10 1 g/100 ml, after a mean duration of treatment of 9 weeks (range 5–32 weeks) The remaining 12 patients with hypocellular marrows did not have pancytopenia in the peripheral blood Eight of these patients showed an improvement in haemoglobin to a mean value of 12 1 g/100 ml after an average of 16 weeks treatment (range 8–28 weeks) Thus 12 of 18 patients in group I were improved after treatment Of these, 10 required no further blood transfusions and the remaining 2 patients, while requiring transfusion, showed a markedly reduced requirement (900 ml blood per week before treatment reduced to approximately 100 ml blood afterwards) These 2 patients belonged to the group with pancytopenia This reduction in transfusion requirement was, in part, due to improvement in platelet count and reduced bleeding tendency 'Post-treatment' haemoglobin concentration was always measured during a period when haemoglobin was relatively constant and at least 4 weeks after the last transfusion

In the other group I patients (those with normocellular or hypercellular marrows) less dramatic but still beneficial responses were noted Two of 4 patients with normocellular marrows showed an increase in haemoglobin concentration of about 3 g/100 ml after 4–5 weeks of treatment Two of 6 patients with hypercellular marrows also showed an increase in haemoglobin concentration In 2 of them whose haemoglobin did not im-

Table III. Therapeutic effect on haemoglobin concentration during oxymetholone treatment

Diagnosis		Number of patients: evaluated	Number of patients: improved	Initial haemoglobin concentration g/100 ml: unimproved patients, median value	Unimproved patients, range	Improved patients, median value	Improved patients, range	Post treatment haemoglobin concentration in improved patients, g/100 ml: median value	Range	Weeks before haemoglobin had increased 3 g/100 ml: median value	Range
Group I	Aregenerative anaemia										
	Hypocellular with pancytopenia	6	4		8.3-8.8	6.8	4.2-7.7	10.1	7.5-10.2	9	5-32
	Hypocellular with neutropenia or thrombocytopenia	12	8	6.4	5.7-10.5	7.6	5.7-10.5	12.1	10.5-13.0	16	8-28
	Normocellular	4	2		7.2-7.5	8.3	8.0-9.0	11.4	10.7-12.1		4-5
	Hypercellular	6	2		6.0-7.0	5.8	4.3-7.3	9.0	7.5-10.5		20-28
Group II	Erythroblastopenia										
	Congenital	3	1	4.5	4.3-4.9	4.9		8.4		16	
	Acquired	3	2	4.9		5.9	5.2-6.9	9.9	9.1-10.8		8-24
Group III	Myelofibrosis	7	1	8.7	6.5-9.9	8.0		12.8		20	

Table IV Therapeutic effect on platelet count during oxymetholone treatment

	Diagnosis	Number of patients: evaluated	Number of patients: with platelet count less than 100,000/µl	Number of patients: improved	Platelet count ×1,000/µl, initial: median value	Platelet count ×1,000/µl, initial: range	Platelet count ×1,000/µl, 'post-treatment': median value	Platelet count ×1,000/µl, 'post-treatment': range	Weeks before platelet count had increased 50,000/µl: median value	Weeks before platelet count had increased 50,000/µl: range
Group I	Aregenerative anaemia									
	Hypocellular with pancytopenia	6	6	2	20	10–30	75	60–90	11	10–12
	Hypocellular with thrombocytopenia	12	8	5	36	21–80	120	86–206	16	14–28
	Normocellular	4	1	1	75		220		6	
	Hypercellular	6	2	0	50					
Group II	Erythroblastopenia									
	Congenital	3	0							
	Acquired	3	1	1	90		500		8	
Group III	Myelofibrosis	7	4	4	42	10–80	150	63–200	16	6–20

Table III. Therapeutic effect of oxymetholone: total material, number of complete and partial remissions

Diagnosis		Number of evaluated cases	Remission complete (CR)	Remission partial (PR)	CR + PR	Improvement (CR + PR) %
Group I	Aregenerative anaemia					
	Hypocellular with thrombocytopenia	6	0	5	5	83
	Hypocellular without thrombocytopenia or thrombocytopenia	12	5	3	8	67
	Normocellular	4	0	2	2	50
	Hypercellular	6	0	2	2	33
Group II	Erythroblastopenia					
	Congenital	3	0	1	1	33
	Acquired	3	0	2	2	66
Group III	Myelofibrosis	7	0	4[1]	4	57

[1] One case temporary CR.

penia a marked reduction in transfusion requirement was observed (from approximately 500 ml blood before, to approximately 175 ml and 100 ml per week respectively, after treatment).

Of 6 patients in group II (erythroblastopenia), 3 improved after from 4 to 24 weeks therapy. In group III (myelofibrosis) only 1 of 7 patients improved. This patient showed a complete, but temporary remission after 20 weeks treatment.

Effect on platelets and granulocytes. 14 of the patients with hypocellular marrow had thrombocytopenia (defined as a platelet count of less than 100 000 cells/µl). Seven of these improved with treatment, showing an increase in platelets from a mean of 25 000 to approximately 100 000 cells/µl (table IV). 14 patients in this group had granulocytopenia (defined as a granulocyte count of less than 1 000 cells/µl). Four of these patients improved after treatment. Mean granulocyte counts rose from 700 cells/µl to stable levels of over 1 000 cells/µl after 13 weeks treatment. The other groups showed no improvement in granulocyte counts.

Of the 7 patients in group III (myelofibrosis) a remarkable improvement on platelet counts was seen in 4. Mean platelet counts rose from 42 000 cells/µl before treatment to 145 000 cells/µl after 16 weeks therapy. Complete remissions were obtained in 5 patients from group I and a

Table VI Oxymetholone treatment, side-effects

Total number of cases studied	[illegible]
Virilisation	[illegible]
Increase in serum transaminases	[illegible]
Jaundice	[illegible]
Amenorrhoea	[illegible]
Oedema	[illegible]
Decreased libido	[illegible]
Hoarseness	[illegible]
Acne	[illegible]
Muscular cramps	1
Headache	1

complete, but temporary, remission in one patient from group III A summary of the therapeutic effect of oxymetholone is given in table V

Side effects of oxymetholone The most frequent side-effects were virilisation and liver damage (table VI) An elevation of serum transaminases was seen in 8 of the 41 patients In 4 there was also a rise in total serum bilirubin The jaundice regressed after temporary suspension of treatment in 2 patients The other 2 patients died (one possibly of serum hepatitis) Jaundice was the only side-effect considered serious enough to warrant withdrawal of the drug

Discussion

The efficacy of treatment of aplastic anaemia has always been difficult to assess Few other studies have attempted to correlate bone marrow cellularity, judged in sections of aspirated marrow, with treatment One such study [7] showed that 57% of patients with genuine aplastic anaemia improved while 22% remained unchanged and 22% died These authors pointed out that before the introduction of oxymetholone, 72% of patients with aplastic anaemia died and only 15% improved GURNEY *et al* [8] have studied the erythropoietic effect of androgens in animals They concluded that the stimulation of erythropoiesis was due to an increased production of erythropoietin but that there was also some evidence of a direct effect on the bone marrow ALEXANIAN *et al* [1] found that erythropoietin excretion increased fivefold after oxymetholone treatment in 75% of 36 adults with various types of anaemia

The results of this study support those of previous studies SANCHEZ-

MEDAL *et al* [11, 12] reported a remission rate of 70% after 2 months treatment. SILINK and FIRKIN [15] showed improvement in 8 of 11 patients (one of whom had myelofibrosis) after from 5 to 12 weeks of therapy. ALLEN *et al* [2] reported 100% improvement in 5 children who had failed to respond to testosterone.

The best results were seen in patients of group I (with hypocellular marrows). Five complete remissions and 8 partial remissions were obtained in the 18 patients in this group. Four partial remissions, including one temporary, complete remission, were obtained in 7 patients of group III (myelofibrosis). Oxymetholone's effect in these patients was predominantly on the platelet counts. We cannot offer any explanation for this. It was noted that even though some patients' haemoglobin concentration did not rise the 3 g/100 ml required to be called a remission, transfusion requirements and frequency of infection were reduced markedly. Once again this was most noteworthy in patients of group I who had hypocellular marrows and peripheral blood pancytopenia. Two out of 4 patients with normocellular marrows and 3 of 6 patients in group II (erythroblastopenia) improved after treatment. Less favourable results were seen in those patients with hypercellular marrows. Such marrow histology has often been found to be preleukaemic in type. This subject has recently been reviewed [10].

The therapeutic effect of oxymetholone seems to be due to stimulation of erythropoiesis, granulopoiesis and megakaryopoiesis in patients with hypocellular bone marrows. It appears that the megakaryopoietic effect predominates in patients with myelofibrosis.

The most serious side effect of oxymetholone in the high doses used in this trial is jaundice. This was the only cause for withdrawal of the drug. Other side effects, though uncomfortable (virilisation, etc.), were mild in comparison to the disease.

We have searched for explanations other than response to treatment for the impressive results obtained in this study. It is possible that the patients studied were a special group. Although no consecutive selection was attempted, we should point out that the early mortality in our study was low in comparison to others' [4].

Many other factors affect the patient type in this form of series. [illegible] high-class [illegible] patients are likely to seek medical aid early and be referred to special centres earlier (perhaps another reason for the low early mortality in this study). The aetiology of the disease is likely to [illegible] In Sweden, for instance, the [illegible]

chloramphenicol is rarely used Broad based laws affecting the use of chemicals in industry will also affect the number of patients admitted to this type of investigation It is to be hoped that as this study continues more light will be shed on these points

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Correlation between Oestriol Levels and Serum Iron-Binding Capacity in Pregnancy

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Abstract. The increase of iron-binding capacity during pregnancy is not influenced by the increased production of oestriol from the fetoplacental unit.

Key Words
Iron-binding capacity
Oestriol
Pregnancy
Serum iron

In pregnancy or after the administration of oestrogens, there is an increase in the serum-binding proteins. It has been demonstrated that throughout pregnancy there is also an increase in unsaturated and total iron-binding capacity [1, 5, 11, 12]. The increase of iron-binding capacity was ascribed to an endocrine factor of the oestrogens [8, 13].

In pregnancy, oestriol showing the highest values among the other oestrogens has the lowest hormonal potency [2, 4, 9, 10]. This investigation has been carried out in order to find the action of oestriol on the serum iron-binding capacity in pregnancy.

Material and Methods

[illegible] binding capacity as well as [illegible] were measured in [illegible] non-pregnant women who were considered as control group and [illegible] pregnant women, [illegible] years old, with normal pregnancy. The gestational age ranged from [illegible] weeks. [illegible] had a normal pregnancy and had [illegible] normal [illegible]

Unsaturated iron-binding capacity (UIBC) was estimated by an isotopic method [illegible] and total iron-binding capacity (TIBC) was estimated by determining the serum iron [illegible]

chloramphenicol is rarely used Broad based laws affecting the use of chemicals in industry will also affect the number of patients admitted to this type of investigation It is to be hoped that as this study continues more light will be shed on these points

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Correlation between Oestriol Levels and Serum Iron-Binding Capacity in Pregnancy

A. Vrettos, J. Mantzos, G. Kokini and E. Gyftaki

Department of Clinical Therapeutics, University of Athens, and Maternity Hospital 'Alexandra', Athens

Abstract. The increase of iron binding capacity during pregnancy is not influenced by the increased production of oestriol from the fetoplacental unit.

Key Words
Iron binding capacity
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Pregnancy
Serum iron

In pregnancy or after the administration of oestrogens, there is an increase in the serum-binding proteins. It has been demonstrated that throughout pregnancy there is also an increase in unsaturated and total iron binding capacity [1, 5, 11, 12]. The increase of iron-binding capacity was ascribed to an endocrine factor of the oestrogens [8, 13].

In pregnancy, oestriol showing the highest values among the other oestrogens has the lowest hormonal potency [2, 4, 9, 10]. This investigation has been carried out in order to find the action of oestriol on the serum iron binding capacity in pregnancy.

Material and Methods

Unsaturated and total iron-binding capacity as well as urine oestriol were measured in 20 non pregnant women who were considered as control group and 55 pregnant women, 20–30 years old, with normal pregnancy. The gestational age varied from 24 to 40 weeks. All had a normal pregnancy and had delivered normal full-term newborns.

Unsaturated iron binding capacity (UIBC) was estimated by an isotopic method using Ironorb-59 (Abbott Radiopharmaceuticals) and total iron-binding capacity (TIBC) was estimated by determining the serum iron [1].

For oestriol measurements a modification of the HUANG [6] method was used. A 24 hour specimen was diluted with water to 2 litres 0.3 ml HCl was added to 2 ml of diluted urine in a Kober tube and the tube was heated in a steam sterilizer at 120 °C for 15 min Oestrone and oestradiol were removed from the cooled hydrolysate by extracting it vigorously with 5 ml of a mixture of benzene light petroleum ether (1 1/v v) for 1 min The tube was centrifuged for 1 min at 500 g and the upper phase was rejected Another 3 ml portion of benzene light petroleum ether solution was added and the tube was gently shaken without mixing the two phases The upper phase was again rejected

The aqueous phase was transferred with 6 ml of water to a separatory funnel and then 3.5 ml of 1 N NaOH and 1 g $NaHCO_3$ were added The oestriol was extracted from the aqueous phase with 25 ml of ether for 3 min Finally oestriol was determined using the Kober reaction and the ITTRICH's extraction technique [7]

Results

In the non pregnant women, TIBC was 342 ± 118 μg%, and UIBC 247 ± 63 μg%. In the pregnant women TIBC was 567 ± 84 μg% and UIBC 408 ± 102 μg%. There is a statistical significant difference in the mean values of TIBC and UIBC between the non pregnant and pregnant women ($p < 0.001$)

Oestriol values in the urine of pregnant women varied between 4.7 and 25 mg/24 h according to the age of gestation Higher values of oestriol were found in late pregnancy

By plotting oestriol values to TIBC values in the group of pregnant women a coefficient of correlation 0.151 ($r = 0.151$) was found which shows that there is no correlation between these values (fig 1)

The serum iron in all cases remained within the normal range

Discussion

According to JEPSON and LOWENSTEIN [8] the increased iron binding capacity in pregnancy may probably be due to the increased production of oestrogens

BURTON [3] found that TIBC was strikingly elevated in women who used oral contraceptives and he suggested that this may be due to the action of oestrogens and or progesterone by producing a rise in β globulins

In this study an increase of oestriol levels was noted not only in UIBC but also in TIBC throughout pregnancy These results are in

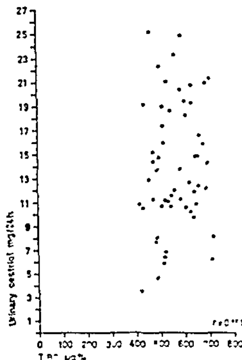


Fig. 1. Comparison of urinary oestriol level and TIBC of 55 pregnant women

greement with the results of other investigators [1–5, 8–10, 12, 13] and ne may expect that TIBC was influenced by oestriol. Since there was o correlation between oestriol and TIBC values and serum iron was ithin the normal range, it is postulated that TIBC in pregnancy is influenced by factors, probably oestrogen fractions, other than oestriol.

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Quantitative Studies of Macrophages in Blood Cultures in Chronic Lymphocytic Leukaemia

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Abstract. Blood cultures in cases of chronic lymphocytic leukaemia showed a low number of macrophages in absence of phytohaemagglutinin (PHA), in addition to a low PHA blastic transformation of lymphocytes. Blood cultures from treated cases developed macrophages in quantities correlated to the grade of PHA blastic transformation and of therapeutic response. A relationship is suggested between the PHA blastic transformation of lymphocytes and the number of macrophages developing *in vitro* without PHA.

Key Words
Blood culture
Lymphocytic leukaemia
Macrophages
Phytohaemagglutinin

PHA treated blood cell cultures develop a high number of lymphoid blast cells, but only a few macrophages [5, 9, 12, 16]. On the other hand, macrophages are numerous in normal blood cultures without PHA [1, 2, 14]. It has been suggested that, in addition to blood monocytes, lymphocytes not engaged in blastoid transformation could be the source of macrophages in culture [2, 6, 8, 15].

It is well known that in chronic lymphocytic leukaemia (CLL) the PHA blastic transformation of blood lymphocytes *in vitro* is low [3, 4, 7, 10, 11, 13], the majority of lymphocytes being unresponsive to PHA. In the present paper we investigated whether a relationship exists between the extent of macrophage appearance in blood cultures without PHA and the percentage of blastic transformation in PHA cultures of lymphocytes from normal subjects and from patients with CLL.

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Key Words
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It is well known that in chronic lymphocytic leukaemia (CLL) the PHA blastic transformation of blood lymphocytes in vitro is low [3, 4, 7, 10, 11, 13], the majority of lymphocytes being unresponsive to PHA. In the present paper we investigated whether a relationship exists between the extent of macrophage appearance in blood cultures without PHA and the percentage of blastic transformation in PHA cultures of lymphocytes from normal subjects and from patients with CLL.

Table I Blast cells and macrophages in blood cultures of normal subjects and of CLL patients

Diagnosis	Number of cases	WBC/mm³	Blastic transformation with PHA, %	Number of macrophages/mm³ without PHA
Normal subjects	17	6,900 (4,600–9,200)	80.06 (65–90) SD 6.09	96.17 (42–210) SD 50.58
CLL untreated	28	84,000 (15,000–400,000)	4.82 (0–17) SD 4.58	2.78 (0–15) SD 4.02
CLL treated with low WBC count	13	12,400 (3,600–22,000)	32.92 (18–50) SD 10.12	69.69 (19–142) SD 42.78
CLL treated with high WBC count	6	38,300 (28,000–56,000)	3.83 (2–5) SD 2.16	5.50 (0–16) SD 7.12

SD = Standard deviation

Materials and Methods

Samples of 15–20 ml of venous blood obtained from 17 normal subjects and from 47 patients with CLL (in 19 cases under treatment) were cultured *in vitro*. The cultures were performed as described by VOLANTE *et al.* [16] in TC 199 (Wellcome) with antibiotics and 15% of autologous serum, so as to obtain a final cellular density of 2×10^6/ml in a total culture volume of 15 ml. In each case, cultures were either treated with 0.02 ml of PHA (Wellcome)/ml of culture, or left untreated. A 18×18-mm glass slide was put at the bottom of the culture tubes according BENNETT and COHN [1]. Blast counts were morphologically performed after 3 days of culture, whereas the number of macrophages adherent to the slides was determined after 6 days. Macrophage count was performed on 8 different areas of 1 mm² and the arithmetical mean determined.

Results

Table I shows that in blood cultures from normal subjects a high percentage of blast cells was observed in the presence of PHA (80.06 ± 6.09%, mean ± standard deviation), and numerous macrophages



Fig. 1. Nuclear blood cell culture from a normal subject: the macrophages are numerous. Giemsa stain, × 1[illegible]

Fig. 2. Nuclear blood cell culture from a case of CLL: few macrophages and lymphocytes are present. Giemsa stain, × 1[illegible]

([illegible] 17 ± 5.136 mm²) in the cultures without PHA (Fig. 1). On the contrary, in untreated CLL patients both a low PHA blastic transformation of lymphocytes (4.52 ± 4.55%) and a scarce development of macrophages without PHA (2.76 ± 4.02 mm²; Fig. 2) were observed. Blasts and macrophages in cultures from CLL patients under treatment were found more numerous in patients with a low white blood cell (WBC) count than in patients with a high WBC count (32.92 ± [illegible].12% and [illegible] ± 42.[illegible] mm² respectively versus 5.61 ± 2.1[illegible],

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CLL treated with low WBC count	13	12,400 (3 600–22,000)	32.92 (18–50) SD 10 12	69 69 (19–142) SD 42 78
CLL treated with high WBC count	6	38 300 (28 000–56 000)	3 83 (2–5) SD 2.16	5 50 (0–16) SD 7 12

SD = Standard deviation

Materials and Methods

Samples of 15–20 ml of venous blood obtained from 17 normal subjects and from 47 patients with CLL (in 19 cases under treatment) were cultured *in vitro* The cultures were performed as described by Volante *et al* [16] in TC 199 (Wellcome) with antibiotics and 15% of autologous serum so as to obtain a final cellular density of 2×10^6/ml in a total culture volume of 15 ml In each case cultures were either treated with 0 02 ml of PHA (Wellcome) ml of culture or left untreated A 18×18-mm glass slide was put at the bottom of the culture tubes according Bennett and Cohn [1] Blast counts were morphologically performed after 3 days of culture, whereas the number of macrophages adherent to the slides was determined after 6 days. Macrophage count was performed on 8 different areas of 1 mm² and the arithmetical mean determined

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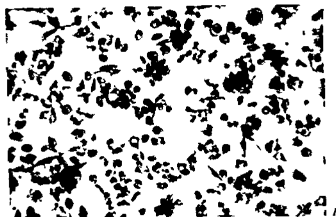




Fig 1 Six-day blood culture from a normal subject the macrophages are numerous. Giemsa stain 100

Fig 2 Six-day blood culture from a case of CLL few macrophages and lymphocytes are present Giemsa stain 100

(96 17 ± 50 58 mm²) in the cultures without PHA (fig 1) On the contrary in untreated CLL patients both a low PHA blastic transformation of lymphocytes (4 82 ± 4 58%) and a scarce development of macrophages without PHA (2 78 ± 4 02 mm² fig 2) were observed Blast cells and macrophages in cultures from CLL patients under treatment were relatively more numerous in patients with a low white blood cell (WBC) count, than in patients with a high WBC count (32 92 ± 10 12%, and 69 69 ± 42 78 mm², respectively, versus 3 83 ± 2 16%,

and 5.50 ± 7.12/mm³). However, the number of macrophages presented marked variations from case to case, as the high standard deviation shows

Discussion

The data show that a relationship exists between the PHA blastic transformation of lymphocytes and the number of macrophages developing in the cultures without PHA. In blood cultures of untreated CLL patients, both the macrophage number and blast cell percentage are very low. After therapy, as others have observed [4, 10], there is an increase of the number of blast cells in the PHA cultures only from subjects with a lowered WBC count, whereas the blast cell percentage remained low in the subjects with a high WBC count: the number of macrophages were high in the former, and low in the latter.

The results do not help in clarifying the debated question whether the macrophages *in vitro* derive from normal lymphocytes or monocytes, since, as shown by HEINE *et al.* [10], in CLL both are diluted by leukaemic lymphocytes. The scarce macrophage production could therefore be related to the low relative number of monocytes and/or normal lymphocytes It can be stated, however, that neoplastic lymphocytes of CLL are undoubtedly unable to transform into macrophages.

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and 5.50 ± 7.12/mm²). However, the number of macrophages presented marked variations from case to case, as the high standard deviation shows.

Discussion

The data show that a relationship exists between the PHA blastic transformation of lymphocytes and the number of macrophages developing in the cultures without PHA. In blood cultures of untreated CLL patients, both the macrophage number and blast cell percentage are very low. After therapy, as others have observed [4, 10], there is an increase of the number of blast cells in the PHA cultures only from subjects with a lowered WBC count, whereas the blast cell percentage remained low in the subjects with a high WBC count: the number of macrophages were high in the former, and low in the latter.

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Acta Haemat 49 340-348 (1973)

A New Anomaly of Platelet Aggregation

A Report of Two Families

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Abstract Two families are described in whom there is an inherited defect of platelet aggregation in response to ADP This consists of a normal response to low concentrations but a reduced response to concentrations adequate to produce complete irreversible aggregation in normal subjects Ten times the usual concentration is required to produce such maximal aggregation Aggregation in response to collagen is normal as is release of ADP in response to collagen This anomalous behaviour is not due to a plasma ADPase This defective behaviour can be duplicated by suitable manipulation of normal platelets and may not occur *in vivo* The existence of this anomalous behaviour contradicts the hypothesis that collagen induced aggregation is mediated by ADP released from platelets This disorder has been named Pearson Stoba anomaly

Key Words
ADP
Collagen
Pearson Stoba anomaly
Platelet disorders
Platelet aggregation
Thrombopathies

Since the classic paper of HASLAM [1] it has been thought that the second wave of platelet aggregation is mediated by ADP released from the platelets themselves by the initial aggregating agent, this ADP in its turn promotes further aggregation Support for this hypothesis has come especially from the study of thrombocytopathies where, in some the lack of aggregation in response to collagen has been demonstrated to be due to a lack of metabolically inactive ADP available for release [2] Recently, however doubt has been cast on the hypothesis [3 4 7] We here report studies on 2 families with a hereditary anomaly of platelet function where collagen must be considered to induce aggregation by some other pathway A brief account of these families has been presented previously [10]

Methods

The methods were as reported previously from this department [6]

Case Histories

Pearson family Propositus I P is the youngest of 4 brothers and presented at the age of 9 with a history of severe epistaxes for 2 years occurring up to 6 times per day and on occasion losing up to 500 ml of blood He has never had abnormal bleeding from any other site and following a successful cautery of the nasal septum at the age of 15 the frequency of the epistaxes reduced dramatically to only 2 per month He has otherwise been extremely fit and is now aged 20 and serving as a police officer

At his first attendance for investigation his haemoglobin was 9 0 g/100 ml and the platelet count whole blood clotting time prothrombin time thromboplastin generation test and Hess test were all normal The only treatment he has received is oral iron with which his anaemia was fully corrected

No other member of the family has ever been troubled by abnormal bleeding and the only haematological abnormalities discovered are those described below (fig 1a)

Stoba family Propositus R S is the elder of 2 brothers and presented at the age of 21 with a 3 month history of haematuria, haemoptysis and possibly haematemesis The only significant past history of severe bleeding was after a dental extraction at the age of 7 All routine haematological investigations were normal as were a chest radiograph, barium meal and intravenous pyelogram After a total period of 6 months the haematuria and haemoptysis ceased and he has subsequently been completely fit and is now serving in the Army No specific therapy was ever given

All other members of the family are well and there is no history of any bleeding disorder (fig 1b)

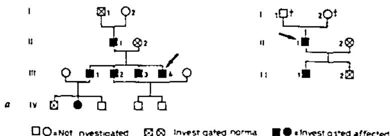


Fig 1 Family trees of Pearson family (*a*) and Stoba family (*b*)

Table I

	Sex	Platelets ×10³ /µl	ADP agg	CTE agg	ADP rel	BT	CR
Pearson family							
I 1	M	222	N	N	N	N	
II 1	M	331	↓	N	N	N	N
II 2	F	225	N	N	N	N	N
III 1	M	309	↓	N	N	N	N
III.2	M	306	↓	N	N	N	N
III 3	M	266	↓	N	N	N	N
III 4, propositus	M	240	↓	N	N	N	N
IV 1	F	502	↓	N	N	N	
IV.2	M	390	N	N	N	N	
Stoba family							
I 1	M	167	↓	N	N	N	N
I 2	F	244	N	N	N	N	N
II 1, propositus	M	234	↓	N	N	N	N
II 2	M	178	N	N	N	N	N

ADP agg = Aggregation with ADP, CTE agg = aggregation with connective tissue extract (collagen), ADP rel = ADP released by aggregating platelets with CTE BT = bleeding time, CR = clot retraction N = normal, ↓ = decreased

Results

The results of the relevant laboratory investigations are set out in table I All results were within the normal range except for the aggregation studies In the cases of the propositi, more extensive investigations were carried out, including prothrombin time, kaolin-cephalin time, and factor VIII assay The results of these tests were normal in all cases

Platelet studies The results of the aggregation studies are illustrated in figure 2 These show that although the response to collagen is essentially normal in terms of the aggregation curves and of ADP release, the response to low concentrations of ADP is at the lower end of the normal range, the normal subject illustrated representing the middle of the range, since 2 µM final concentration (f c) is required to produce definite aggregation and disaggregation, only reversible aggregation occurs at 20 µM

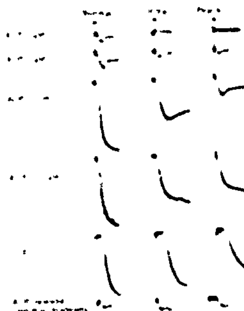


Fig. 2. [illegible]

[illegible] and approximately [illegible] was required to produce a curve of maximal velocity comparable to that obtained in all normal subjects with [illegible]. There is a [illegible] in velocity of response as indicated by the steepness of the slope of the curve over the range [illegible]. In contrast, in the normal subject, the maximal velocity has already been achieved at [illegible]. The response to the [illegible] was normal.

One possible explanation for the reduced response to ADP could be the presence of an ADPase in the plasma. To test this hypothesis, platelet-rich plasma was obtained from a normal subject, the concentration of platelets in which was 450 000/µl. This was then diluted 1:1 with platelet-poor plasma obtained from (a) same donor, (b) Pearson family member, (c) Sh[illegible] family member. Each mixture was then incubated at 37 °C for 5 min and 1 ml aliquots transferred to the aggregometer and aggregated with ADP [illegible] in the usual way. The resulting curves are illustrated in Figure 3 and are clearly identical.

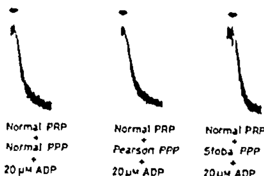


Fig 3 Aggregation curves demonstrating absence of an ADPase (see text)

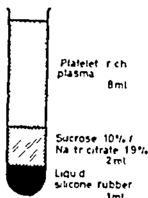


Fig 4 System for concentrating platelets

Whilst developing a method for concentrating platelets in order to study platelet aggregation in cases of thrombocytopenia [8, 9], certain observations were made which may be relevant. Platelet-rich plasma from a normal subject was spun in the system illustrated in figure 4 for 60 min at 2,400 rpm, i e 6 times as long as is employed for concentrating platelets in thrombocytopenia and after centrifugation the supernatant was pipetted off and the platelets on top of the silicone rubber could be resuspended in platelet-poor plasma to a concentration of 300 000 /μl and aggregation studies performed. The resulting curves are illustrated in figure 5 and show far closer resemblance to those obtained with platelets of affected members of these families than to those obtained with the same normal subject's platelets before centrifugation. The effects of the procedure seem perhaps to be too severe in that after centrifugation aggregation is not complete even at ADP 200 μM f c

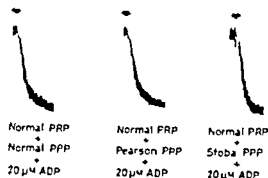


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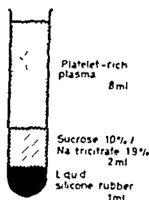


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6-month episode, not resembling the presentation of any haematological cause of bleeding and which remitted without any therapy Of the 13 members of the 2 families investigated, a total of 8 were shown to possess the anomaly, of whom 6 never had any bleeding problem We may postulate that this anomaly which appears to be inherited as an autosomal dominant characteristic, is of no significance in normal haemostasis and is only demonstrable under experimental conditions The existence of such traits is well known, the classic example being the ability to taste phenyl thiocarbamide

Although the anomalous behaviour of platelets in the affected members of these 2 families is to some degree an artefact, there must exist in these subjects an alternative pathway for collagen induced aggregation It may be doubted whether this alternative pathway plays any part in haemostasis but under experimental conditions it is clearly demonstrable The nature of this pathway is quite unknown

That this is a new anomaly is evident when it is compared with other qualitative platelet disorders Glanzmann's thrombasthenia shows no aggregation with either ADP or collagen and thrombocytopathy shows a normal response to ADP but virtually no response to collagen The Sprowson anomaly [6] is the reverse of the situation described here in that the response to collagen is normal but there is an exaggerated response to ADP The Goddard anomaly [8] combines features of both thrombocytopathy and the present anomaly in that there is a diminished response to ADP combined with virtually no response to collagen It is of interest to note that the Goddard anomaly which is probably inherited as a single autosomal dominant trait combines the 2 features of diminished response to ADP and lack of response to collagen which are themselves inherited as independent autosomal dominant traits

Following the normal custom of naming abnormalities after the first patients described, we offer the name 'Pearson-Stoba anomaly', for the condition described here

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Thrombocytopathia with Abnormalities in Platelet Release Reaction

Some Evidence for Platelet Factor 4 Deficiency

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Abstract The prolonged bleeding time in a 70-year-old man with a bleeding diathesis was attributed to impaired platelet aggregation This could be accounted for by the decrease of the platelet adhesiveness to collagen and the decreased release of adenosine diphosphate (ADP) The impaired availability of platelet factor 3 and acid phosphatase after induction by collagen was partly corrected by ADP or bovine fibrinogen The total activity of platelet factor 3 and acid phosphatase was however normal in contrast to the lowered total activity of ADP and platelet factor 4 which showed even a disturbance of their release from platelets without any correction in the presence of ADP Similarly the total activity of β glucuronidase was normal though its release following collagen induction was impaired

Key Words
Bleeding disorders
Platelet biochemistry
Platelet factors 3 and 4
Platelet function
Thrombocytopathia

Primary arrest of bleeding is effectuated by a venous reaction not yet exactly defined platelet aggregates being formed on the spot of the lesion [22] The formation of tightly packed, irreversible aggregates is one of the most important properties of the blood platelets and a prerequisite for their function in the hemostatic process

Circulating blood platelets rapidly adhere to the area of trauma, to collagen [17] and other substances [2] Following their adhesion, a specific and rapid release of adenosine diphosphate (ADP) and other substances from platelets occurs [5] Released ADP produce platelet aggregation and is also necessary for making platelet factor 3 (PF 3) available for its role in the intrinsic coagulation mechanism [10, 22]

Recently, patients with bleeding disorders have been reported in whom the prolonged bleeding time has been attributed to abnormal platelet-collagen reaction [4, 11, 13, 26, 31, 32]. The disease is charaterized by an impaired collagen-induced platelet aggregation, and PF 3 availability. In some of these cases a specific defect in the release of platelet ADP has been found [11, 31, 32].

We have recently demonstrated 3 patients of the same family with bleeding disorders in whom defects in collagen-induced aggregation, release of ADP and PF 3 availability were accompanied by an impaired platelet acid phosphatase availability [20]. In the present paper we have followed another patient, in whom the defect in the collagen-induced aggregation and ADP release was accompanied by an impaired platelet factor 4 (PF 4) release with a decrease of its total content in the platelets.

Case Report

P.A. is a 70-year-old man with a lifelong history of easy bruising to which he has not paid any attention for a long time. Since 1971 the bleeding syndrome began to aggravate. Blood suffusions, localized especially on the upper extremities and the trunk, were so intense that they lifted the skin above the level of the non-affected areas. Suffusions formed even in the mouth and in the nose. Similarly epistaxes gained in intensity.

Laboratory examinations revealed a posthemorrhagic anemia of medium degree and a prolonged bleeding time, the number of platelets being normal. The basic biochemical parameters were within the limits of physiological values.

Routine coagulation studies including factors VIII, IX, V, VII+X, XIII, thrombin time, fibrinogen and fibrinolysis in euglobulins were normal. Besides the prolonged bleeding time there was even a slightly decreased resistance of capillaries and an impaired consumption of prothrombin.

The family history of the patient was negative with regard to bleeding.

Materials and Methods

Blood for platelet studies was obtained by mixing 9 parts of venous blood with one part of either 3.8% trisodium citrate or a solution containing 1% EDTA Na_2 and 0.7% saline. Platelet rich plasma (PRP) was prepared by centrifuging blood at 280 g for 15 min at 16 °C. The platelet count was performed using PITTY's method [24]. Platelet poor plasma (PPP) was prepared by centrifugation of PRP at 16 000 g for 20 min at 12 °C.

Platelet suspensions were prepared according to CRONBERG and CAEN [6]. Blood was drawn in EDTA Na_2, and after differential centrifugation the platelet

button was resuspended by gentle pipetting in 25 ml of a wash solution consisting of 2 parts of 0.077 M EDTA Na_2 and 98 parts of 0.130 M NaCl, 0.005 M KCl and 0.015 M Tris adjusted to pH 7.35 with 1 N HCl. The platelet suspension was recentrifuged at 1 000 g for 12 min at 16 °C. The washing was repeated and the platelets were resuspended in the buffer solution used in the washing omitting EDTA Na_2.

Purified bovine fibrinogen (LST Co., England), collagen (Stago, France), ADP (Boehringer, Mannheim), adrenaline (Spofa, Czechoslovakia), stypven (Burroughs Wellcome, London) were used and diluted to the required concentrations by adding Michaelis buffer at pH 7.35.

Glass bead retention was performed by the method of VORLICKÝ *et al.* [30].

EDTA Na_2-PRP was used to determine the adhesion of platelets to connective tissue [31].

PF 3 availability was tested by a modification of the method of SPAET and CINTRON [29]. 0.1 ml of the test material was collected from a siliconized test tube at intervals of 0, 5, 10 and 20 min and transferred into another test tube containing 0.1 ml of Michaelis buffer at pH 7.35 and 0.1 ml of stypven reagent (10 µg/ml). 0.1 ml M/40 solution of $CaCl_2$ was added immediately to the test material and the coagulation time of the sample was measured. The PF 3 available after induction was expressed as a percentage of the total PF 3. The latter was defined as 100% at same volunteer, a dilution curve was obtained for each individual. Quantitative determination of PF 3 activity was also tested according to POLÁŠEK and DUCKERT [25].

The PF 4 release and its total activity were tested by means of the heparin thrombin time. The details of the procedure will be presented elsewhere [18].

The availability of acid phosphatase in the form of paranitrophenylphosphatase was determined according to the method of KUBISZ and CAEN [19].

β-Glucuronidase in the form of phenolphthalein β-D-glucuronidase was determined by a modification [8] of the method of FISHMAN [9].

The total amount of ADP and the amount of ADP released from platelets after incubation with collagen were determined enzymatically [1] using the reagents of Boehringer, Mannheim (ADP/AMP test, Cat. No. 15980 TAAB).

Platelet aggregation was measured according to a technique based upon the turbidimetric method of BORN and CROSS [3], with an apparatus permitting continuous stirring, but not automatic recording.

Table I. Initial data on primary hemostasis

	Patient	Normal values mean ± 2 SD
Platelets, × 10³ mm³	180	170–380
Ivy bleeding time, min¹	30, 30	2–8
Retention to glass beads, %¹	5, 10, 6	30–70
Adhesion to connective tissue, %¹	2, 1, 0	14–24

¹ Values obtained on different days

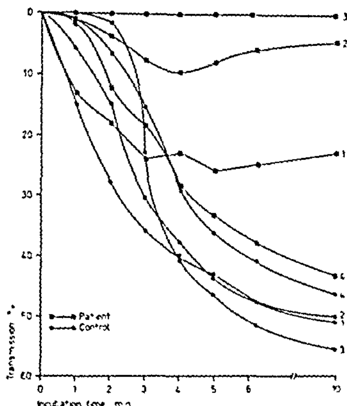


Fig. 1 Platelet aggregation induced by ADP 4×10^{-6} M (1), adrenaline 6×10^{-6} M (2) collagen 20 μg/ml PRP (3) bovine fibrinogen 0.4 mg/ml PRP (4)

Results

Primary hemostasis. As is evident from table 1, besides a prolonged bleeding time the adhesiveness of platelets to glass and their adhesion to collagen were decreased. The number of platelets and their morphology as well as the retraction of the clot were normal.

Platelet aggregation. ADP-induced aggregation at room temperature was normal but at 37 °C the initial wave of aggregation produced by 4×10^{-6} ADP was consistently followed by disaggregation. Aggregation of platelet by bovine fibrinogen (0.4 mg/ml) was normal. The first wave of epinephrine-induced aggregation was normal, but the second wave was always smaller than observed in normal subjects or absent.

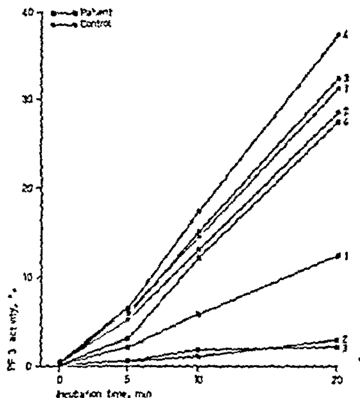


Fig 3 Availability of platelet factor 3 induced by ADP 4×10^{-6}M (1), adrenaline 6×10^{-6}M (2), collagen 20 µg/ml (3), bovine fibrinogen 0.4 mg/ml (4)

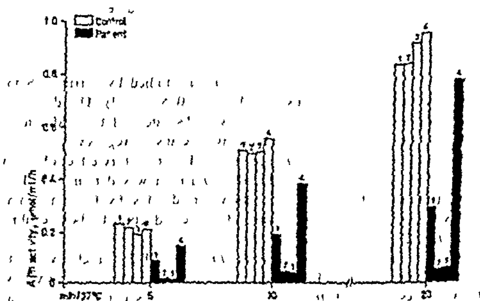


Fig 4 Availability of platelet acid phosphatase induced by ADP 4×10^{-6}M (1) adrenaline 6×10^{-6}M (2), collagen 20 µg ml (3) bovine fibrinogen 0.4 mg ml (4)

Table II The amount of platelet factor 4 (PF 4) in the platelets and its release induced by ADP (4×10^{-6}M), adrenaline (6×10^{-6}M) and collagen (20 μg/ml)

	Release of PF 4 induced by			Total amount of PF 4 in platelets
	ADP	adrenaline	collagen	
Normal subjects, mean ± SD (n = 26)	42 ± 6	42 ± 6	67 ± 9	100 ± 21
Patient	0.5	0.7	0.9	42

Table III Platelet β-glucuronidase and its release induced by collagen (20 μg/ml). The results are shown as e.u 10 platelets

	Total amount of β-glucuronidase in platelets	β-Glucuronidase released to supernatant
Normal subjects, mean ± SD (n = 11)	0.80 ± 0.12	0.15 ± 0.05
Patient	0.82	0.03

Table IV Platelet ADP and its release induced by collagen. Amount of ADP in platelets and released into supernatant by collagen (20 μg/ml) are shown as μmol/10 ¹ platelets

	Total amount of ADP in platelets	ADP in platelets after induction by collagen	ADP released to supernatant
Normal subjects, an±SD	2.9 ± 0.6	[illegible]	1.5 ± 0.4
ent	1.1	0.8	0.05

Mean values of controls [8]

Similarly, after the induction by collagen or adrenaline, the activity of acid phosphatases was almost immeasurable, but in the presence of ADP or bovine fibrinogen there was an increasing activity, yet even here the values were somewhat smaller than in the controls (fig 4) The examinations of the total activity of PF 3 and acid phosphatase showed normal values

Amount of PF 4 in platelets and its release As is evident from table II the total activity of PF 4 was diminished Its release after the induction by ADP, adrenaline, bovine fibrinogen or collagen was almost zero

Amount of ADP and β-glucuronidase in platelets and their release The amount of ADP in the platelets and released into supernatant by collagen stimulation was markedly diminished (table III) The level of β-glucuronidase in the platelets of the patient was normal, but its release after the induction by collagen was decreased (table IV)

Discussion

The prolonged bleeding time in connection with the bleeding syndrome shown in the patient is in accordance with a defect of primary hemostasis which seems to be caused by an abnormal interaction of platelets with collagen The aggregation of platelets after the induction by collagen requires the release of ADP, which takes place after the adhesion of platelets to the collagen fibrillae The disturbed adhesion to collagen and the impaired release of platelet ADP can thus be considered to be the basic defect The release of ADP is also necessary for the production of the second wave of aggregation following the induction by adrenaline [23] bovine fibrinogen or a smaller dose of ADP and is of importance for the activation of PF 3 and acid phosphatase [10, 19]

The process, by which collagen and other substances induce the rapid discharge of the content of dense bodies and α-granules from the platelets to the extracellular environment was denoted as a platelet release reaction' and divided by HOLMSEN *et al* [14] into the phases of induction, intracellular transmission, and of extrusion A defect of some in these phases may then be responsible for the disorder of aggregation after the induction by collagen

Having examined the metabolism of adenine nucleotides in 6 patients with an impaired aggregation after the induction by collagen connected with a disorder of the release reaction HOLMSEN and WEISS [16] have

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from the platelets had no effect on the availability of PF 3 or acid phosphatase [21] These observations may as well point to the possibility that PF 4 is localized in the dense bodies If this hypothesis were correct the determination of the total content of PF 4 and its release from the platelets might serve as a simple complementary test for a more accurate classification of thrombocytopathias with an impaired release reaction

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A. G. STEINBERG and A. G. BEARN (ed.) Progress in Medical Genetics, vol 8. Grune & Stratton, New York 1972. 319 pp.

Each year, the new volume of 'Progress in Medical Genetics' consists of a number of chapters of the highest level. As in former volumes, the seven articles of the present volume vary from the treatment of general topics to that of more specialized ones. Among the former is FRANK FENNER's comprehensive treatment of 'Genetic Aspects of Viral Diseases of Animals' and NEWTON E. MORTON's sweeping essay on 'The Future of Human Population Genetics'. Among the specialized articles, that by CHARLES I. SCOTT, Jr., presents a revision of the taxonomy of 'The Genetics of Short Stature' as encountered in several syndromes, with emphasis on the great phenotypic heterogeneity of the various malformations formerly often treated as if they were a single class. Biochemical progress is the subject of HENRY N. KIRKMAN's informative report on 'Enzyme Defects' and ROSCOE O. BRADY's and EDWIN H. KOLODNY's detailed analysis of 'Disorders of Ganglioside Metabolism'. The old problem of the causal aspects of chromosomal abnormalities and cancer are impressively dealt with by JAMES GERMAN in 'Genes which Increase Chromosomal Instability in Somatic Cells and Predispose to Cancer'. The discussion includes the effects of four genes, one of which being that for Bloom's syndrome, that determine not only abnormal chromosome behavior but also severe clinical abnormalities. Finally, C. A. CLARKE's *monographic chapter on 'Prevention of Rh Isoimmunization' summarizes* the work which resulted in one of the greatest beneficial accomplishments of medical genetics.

Each article will be read with benefit both for the facts known in its area and for the tasks ahead. The generous space taken up by the references is one of the valuable properties of the book. (Praise is due to the publisher for returning to the useful way of printing the references so that the name of the author or senior author stands out at the left of each page.) C. STERN, *Berkeley, Calif.*

S. SELL. Immunology, Immunopathology, and Immunity. Harper & Row, London 1972. 277 pp., ind., 45 fig., US $ 12.95.

Quite a few text-books of immunology student and immunopathology exist; however, each time a medical or a biology student asks for the title of a concise introduction to these fields, one is embarassed. The book of Prof. SELL fills this gap beautifully. Both the protective and destructive mecanitm of the immune system are presented in three parts. The first part 'Immunology' contains the induction and expression of immune reactivity. The mecanisms and effects of immune reactions in the propagation of tissue lesions and diseases (allergic reactions) are the subject of part 2, 'Immunopathology'. The last part, 'Immunity', covers the world of immune reactions in protecting against infections and cancer. Reading these 277 clearly printed pages, one gets an idea as clear and concise as is possible today about everything which is important in immunology. The value of this introductory text is still increased by the fact that all statements and reprinted items are fully covered by references to the pertinent original litterature and by the excellent subject index. This book is widely recommended and is a must for every teaching library.

T. L. VISCHER, *Geneva*

J. BERNARD et J. RUFFIE (éd.) L'Hématologie Géographique. Comporte deux volumes subdivisés chacun en deux parties, vol. 2. Masson, Paris 1972. 358 pp., 61 fig. fFr 110.–.

Le premier volume avait été consacré dans sa première partie à l'étude des milieux de vie humains et à leurs rapports avec l'hématologie et, dans sa deuxième partie, à l'hémotypologie c'est-à-dire aux facteurs sanguins divers (groupes érythrocytaires, leucoplaquettaires, sériques, enzymes globulaires et plasmatiques, hémoglobine) sous contrôle génétique mais soumis également, dans leur répartition et leurs déviations, au milieu extérieur.

Le deuxième volume se compose des troisième et quatrième parties de longueur inégale. La troisième partie d'environ 300 pages a pour titre «Les anomalies hématologiques acquises» et s'intéresse aux anomalies conditionnées par le milieu de façon directe: les éosinophilies, les anémies, les leucémies.

1. La description, la régulation, les fonctions des éosinophiles ainsi que les caractères de l'éosinophilie parasitaire sont précisés avant l'étude des diverses parasitoses en cause et de leur répartition géographique. Les éosinophilies allergiques et les autres éosinophilies complètent ce chapitre qui propose de la sorte une mise au point complète sur l'éosinophilie en général.

2. Les anémies acquises liées aux facteurs géographiques.

a) Les anémies parasitaires qui sont classées selon le mode d'action du parasite responsable en anémie par atteinte directe du système réticulo-endothélial, anémie par atteinte directe du globule rouge circulant, anémie par spoliation sanguine, anémie par troubles métaboliques.

b) Les anémies nutritionnelles liées à des carences alimentaires. Le rappel des facteurs de l'érythropoïèse sert de base à la classification des formes cliniques: anémies protéiprives, par carence en acide folique et vitamine B12, par carence martiale (chez l'homme et les animaux), par carences multiples. Leur répartition géographique dépend de très nombreux éléments, climat, conditions sociales et économiques, traditions ethniques, sans oublier la combinaison avec les facteurs parasitaires.

A propos des anémies dans diverses régions du globe est fournie une documentation d'une extrême richesse scientifique pleine de connaissances vécues, souvent pittoresque sur les conditions de vie qui engendrent ces états pathologiques et sur l'influence des événements mondiaux qui en ont fait naître certains.

La maladie du Kwashiorkor est considérée à part comme maladie d'actualité demeurant en permanence un responsable potentiel de «véritable génocide».

c) Les anémies d'origine toxique sont provoquées par des produits de plus en plus nombreux. Des tableaux récapitulatifs en dressent les listes, les doses tolérables qu'il s'agisse de substances à usage agricole ou alimentaire. Les effets toxiques médicamenteux, leur mécanisme, leur origine sont répertoriés et étudiés: des tableaux en donnent également les noms, apportant les indications utiles que le thérapeute cherche si souvent et trouve si péniblement en raison de leur dispersion habituelle.

3. La connaissance des rapports entre les leucémies et les conditions écologiques est certainement capable d'apporter des notions capitales sur ces maladies. Mais les moyens diagnostiques manquent encore en beaucoup de régions médicalement sous-équipées. Néanmoins les données disponibles présentent un très vif intérêt. Ces

renseignements échappaient en général à l'hématologiste aussi le regroupement qui lui est ici présenté, est il particulièrement intéressant de même que la revue générale des rapports entre la génétique et les leucémies et la revue de l épidémiologie des leucémies animales

L ensemble de cette troisième partie de l ouvrage represente d abord un traité hématologique de base actuel complet approfondi sur les sujets qui s y trouvent inclus Il peut être utilisé comme une source de renseignements, très riches très diversifiés, très nouveaux et sans équivalent pour ces chapitres sa valeur documentaire et didactique est considérable

Tout en gardant une présentation classique des données ce qui permet de l utiliser *comme un excellent livre d hématologie* cet ouvrage apporte en plus un point de vue entierement nouveau Les auteurs décrivent et font voir au lecteur non seulement les maladies mais aussi les hommes qui en sont frappés Est constamment évoquée en meme temps que les agents étiologiques, la variabilité de leurs conséquences en fonction des individus de leur mode de vie de leur origine génétique

Le facteur géographique est entendu dans un sens très large puisqu il comporte une masse énorme de connaissances historiques anthropologiques, économiques et meme politiques La relation de ces facteurs avec l hématolog e est soumise à une analyse pénétrante où se révèlent non seulement la haute compétence des auteurs mais aussi une conception générale de l homme en fonction du monde et de l évolution de l espèce

La dernière et quatrième partie en une cinquantaine de pages développe ces thèmes et trace en un élargissement de l hématolog e géographique l évolution des hominiens sans quitter les amarres de la réalité exacte et scientifique elle entraîne véritablement à une réflexion philosophique sur le destin de l'homme

S MAYER et R. WAITZ, *Strasbourg*

P RENTCHNICK (ed) Recent Results in Cancer Research, vol 39 Current Problems in the Epidemiology of Cancer and Lymphomas Symp Ges Bekämpfung Krebskrankheiten Nordrhein Westfalen Dusseldorf 1971 ed bei E. GRUNDMANN und H TULINIUS Springer Berl n 1972 VIII + 248 pp 80 fig DM 58 –/US $ 18 40 ISBN 3-540-05560-5

Studien zur Epidemiologie der Krebskrankheiten konnen sich sehr unterschiedlich präsentieren als detaillierte Morbiditäts und Letalitatsstatistik als histologischer Klassifikationsversuch in Relation zum Krankheitsverlauf als Versuch soziale ethnische ökologische und geographische Faktoren mit Morbiditatsziffern in Verbindung zu bringen schliesslich als d rekte Suche nach Karzinogenen darunter vor allem nach infektiösen Agent en vorab nach Viren Entsprechend der Vielfalt dessen was unter dem Begriff «Epidemiologie» verstanden werden kann erscheinen d e Beitrage dieses Bandes Jeder Beitrag ist als Einzelpubl kation rei hlich mit Abbildungen Tabellen und Literaturzitaten versehen gedacht Übersichtsartikel wechseln mit Originalarbeiten experimentellen Charakters ab Wie aus der unten aufgeführten, den Inhalt des Buches am besten charakterisierenden Liste der *einzelnen Beitrage* ersi htli h ist, wird mi t Ausnahme der Lymphome auf Zusammerhänge zwischen Haufigkeit einzelner Tumoren und infektiösen Erregern nicht

eingegangen Auch wird zugunsten neuerer Erkenntnisse und Theorien auf Bekanntes aus der Krebsepidemiologie verzichtet, so dass dieses Buch auch dem epidemiologisch Interessierten durchaus neue Perspektiven zu offnen vermag

Die Titel der Abhandlungen sind folgende Das Programm der WHO zur histopathologischen Definition und Klassifikation von Tumoren, Vorlaufige Resultate einer Untersuchung zur epidemiologischen Signifikanz detaillierter Tumordaten Die Vielfalt der Lungentumoren Klassifikationen Die Signifikanz von Asbestkorperchen im Gewebe Die histologische Klassifikation des Schilddrüsenkarzinoms als epidemiologisches Problem Leukamie und Schilddrusenkarzinom bei Atombomben überlebenden von Hiroshima Tumoren des Gastrointestinaltraktes bei Immigranten. Histologische Form des Magenkarzinoms in Beziehung zur intestinalen Metaplasie Primäres Leberkarzinom in Hongkong mogliche atiologische Faktoren Neue Möglichkeiten und Hypothesen der Epidemiologie des Leberkarzinoms Aflatoxine und Leberkarzinom beim Menschen. Das latente Prostatakarzinom Mortalitat und Morbiditat des Prostatakarzinoms. Die Haufigkeit des Prostatakarzinoms eine epidemiologische Übersicht. Epidemiologische Studien zur histologischen Differenzierung des Peniskarzinoms Die Karzinogenese des Zervixkarzinoms als epidemiologisches Modell Hormone in der Ätiologie und im klinischen Verlauf des Mammakarzinoms Die Rolle der Ovarien bei der Entstehung des Mammakarzinoms. Primäres intestinales Lymphom klinische Manifestation und möglicher Einfluss von Umgebungsfaktoren Entdeckung und Bedeutung von Gruppenerkrankungen an Burkitt s Lymphom und Morbus Hodgkin Epidemiologische und immunologische Faktoren in der Pathogenese des Burkitt Tumors Die Ätiologie des Burkitt Lymphoms Epidemiologie des Morbus Hodgkin Morbus Hodgkin bei Kindern eine epidemiologische Studie in Norddeutschland.

G A NAGEL, *Basel*

Pappenheim-Preis 1973

Anlässlich der 79. Tagung der Deutschen Gesellschaft für Innere Medizin verlieh die Deutsche Gesellschaft für Hämatologie den Pappenheim-Preis 1973, der von den Nordmark-Werken Hamburg mit einem Geldbetrag dotiert wird, an Dr. GÖTZ KAISER, Dr. DIETRICH GAUGER und Dr. KLAUS QUIRING (Zentrum der Pharmakologie, Klinikum der Universität Frankfurt) für ihre Arbeit «Der Retikulozyt als zelluläres Modell eines adrenergischen β-Rezeptor-Effektor-Systems».

International Society of Haematology

The 3rd Meeting of the European and African Division of the International Society of Haematology will be held in London at the Royal Festival Hall, on August 24–28, 1975. President: Prof. J. V. DACIE. Secretary: Dr. S. M. LEWIS.

The scientific programme will include reviews, presentation of new work, round table panel discussions on pratical aspects of the subjects and workshops on specialized topics. The main sessions will be on the following topics: hereditary haemolytic anaemias, thrombosis and haemostasis, immuno-haematology, erythropoiesis, aplastic anaemia, leukaemia, haemoglobin, thalassaemia, haemoglobinopathies, nutritional anaemias, lymphomas, myeloma.

There will be an exhibition of technical equipment, laboratory instruments and pharmaceutical products. There will also be scientific demonstrations and scientific film shows.

The official language of the meeting will be English.

Further information can be obtained from: Dr. S. M. LEWIS, Department of Haematology, Royal Postgraduate Medical School, Ducane Road, *London, W12 OHS* (England).

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Bearbeitet von G. Boehm, Basel

(B) = Book reviews – Buchbesprechungen – Livres nouveaux

Pappenheim-Preis 1973

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